Characterisation of pyrolysis mass spectrometry for use in marine algal systematics

Thesis submitted in candidature for the degree of Doctor of Philosophy

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Abstract

Pyrolysis mass spectrometry (PyMS) is a rapid, automated analytical technique that is used for chemical and biological characterisation of organisms. It has been limited in its use outside the discipline of microbiology and has rarely been applied to the analysis of multi-cellular organisms. This study aimed to investigate the potential of using PyMS as a routine analytical tool to resolve problems in marine algal systematics. The technical constraints of PyMS were also examined.

The effect of sample concentration proved to be an important consideration for the production of meaningful results. PyMS analysis of macroalgae from the order Fucales demonstrated that this technique was robust to the influence of environmental variability and challenged the assertion that it is limited to use as a phenotypic technique only. Characterisation of samples was also possible at the sub-species level. Experimentally induced variation among cultures of the diatom *Skeletonema costatum*, including silicate limitation, low salinity and reduced irradiance, was detectable by PyMS.

PyMS is subject to technical limitations including day to day variability among spectral data and does not produce a permanent classification. This study showed that PyMS is a highly discriminatory, sensitive technique that is capable of resolving chemical and biological variability among marine algae.

Acknowledgements

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Finally I would like to dedicate this thesis to my loving, supportive family, Mum, Dad, Fraser, Lynn and Stephen, and to thank them for always being there and for believing in me.

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Contents

Glossary

Analytical pyrolysis	The characterisation, in an inert atmosphere, of a material or a chemical process by a chemical degradation reaction(s) induced by thermal energy
ANCOVA	Analysis of covariance
ANNs	Artificial neural networks
ANOSIM	Analysis of similarity
CCAP	Culture Collection of Algae and Protozoa
Curie-point	Temperature at which the magnetic property of any ferromagnetic material disappears
Curie-point pyrolyser	A pyrolyser in which a ferromagnetic sample carrier is inductively heated to its Curie-point
CVA	Canonical variates analysis
EI	Electron impact
F/2 + Si	Guillard's medium for diatoms
G	Gravity
GLM	General linear model
HCA	Hierarchical cluster analysis
ml	Millilitre
mm	Millimetre
m:z	Mass:charge
PAR	Photosynthetically active radiation
PC	Principal component
PCA	Principal components analysis
PC-CV	Principal components – canonical variates
PSU	Practical salinity unit
PyGC	Pyrolysis gas chromatography: a pyrolysis technique in which volatile pyrolysates are directly conducted into a gas chromatograph for separation and detection
PyMS	Pyrolysis mass spectrometry: a pyrolysis technique in which the volatile pyrolysates are detected and analysed by on-line mass spectrometry
Pyrolysate	Low molecular weight fragments of sample resulting from pyrolysis mass spectrometry

RaPyD	Rapid Automated Pyrolysis Discriminator
S_G	Gower's similarity coefficient
µE	Micro-Einstein
µg	Microgram
µl	Micro-litre
µM	Micro-molar

Chapter 1

General Introduction

1.1 Introduction

Systematics is the scientific study of the types and diversity of organisms and of relationships among them including identification, classification, evolutionary processes and genetic mechanisms (Goodfellow & O'Donnell, 1993). Biological classifications have two major objectives; firstly, they form the basis of biological generalisations in comparative studies, and also serve as the key to information storage systems (Mayr, 1994). The development of novel techniques for the rapid, accurate and automated characterisation and quantification of experimental organisms aids progress in biological research. In recent years, novel analytical techniques, including cytological methods (Sukenik & Carmeli, 1989), biochemical and physiological techniques (Kessler *et al.*, 1996), and molecular genetic analyses (Peters *et al.*, 1997; Rousseau & de Reviers, 1997; Serrão *et al.*, 1999), have been developed for use in studies of systematics and evolution of marine algae.

Among marine macroalgae, particularly the order Fucales, two phenotypic conditions that have caused great taxonomic difficulty exist. These are the developmental state of the plant, which may reflect chemical as well as morphological differences, and its responses to habitat conditions (Russell, 1978). Further confusion in algal systematics also arises where morphologically similar species are regarded as a single entity (Bird & van der Meer, 1992). Failure to recognise the full extent of morphological variation has sometimes led to narrow species concepts and unclear distinction between species with similar forms (Bird & van der Meer, 1993). Physiological and biochemical characters also vary in algae and have sometimes been misinterpreted in characterisation and assessment of economically important species (Craigie *et al.*, 1984). The importance of rapid and accurate characterisation of algal species is

highlighted, for example, by research of toxin-producing marine phytoplankton which cause harmful algal blooms (e.g. Takahashi & Fukazawa, 1982). More precise delimitation of seaweed species and better identification of toxic and closely related non-toxic phytoplankton have been identified as areas for development in algal systematics (Bird & van der Meer, 1992). The development of better tools with which to address these issues is also important.

Chemotaxonomy is the study of chemical variation in living organisms and the use of chemical characters in classification and identification may support traditional taxonomic methods (Goodfellow & Minnikin, 1985). Pyrolysis mass spectrometry (PyMS) is a rapid instrument-based technique, which has been used for the analysis of complex organic materials (e.g. Meuzelaar *et al.*, 1974) and may be of value in chemotaxonomy.

This chapter describes the development of pyrolysis mass spectrometry (PyMS) as an analytical technique and briefly reviews some of its historical and present applications.

1.2 PyMS general principles

Pyrolysis has been defined as thermal degradation, in a non-oxidative environment, resulting in the formation of volatile low molecular weight fragments (pyrolysate) (Levy, 1966). The pyrolysate products reflect the composition of the original sample and can be separated and quantified by gas chromatography (PyGC) or mass spectrometry (PyMS) to give characteristic information on the original material (Wieten *et al.*, 1984). As an analytical system, PyMS has some advantages over PyGC in that it is faster, more reproducible and more easily automated (Meuzelaar & Kistemaker, 1973; Aries *et al.*, 1986). Individual components within the pyrolysate are not identified, but data are presented as a mass spectrum of the pyrolysis products

and the appearance of this 'pyrogram' can be used as a unique biochemical 'fingerprint' for each sample (Magee, 1993).

1.3 Development of the technique

Zemany (1952) first demonstrated the use of pyrolysis in combination with mass spectrometry as a taxonomic technique. He discovered that under standardised pyrolysis conditions, complex organic material degraded reproducibly, forming products that were characteristic of the original sample. The development of a fully automated pyrolysis mass spectrometer (Meuzelaar & Kistemaker, 1973) created the potential of this instrument as a routine analytical technique, initially for samples such as bacteria (e.g. Meuzelaar *et al.*, 1984). Meuzelaar *et al* (1984) demonstrated the use of PyMS in combination with computerised multi-variate statistical analyses for the analysis of a range of materials, from simple synthetic polymers to whole microorganisms such as *Escherichia coli*. Several reviews on the use of pyrolysis for the analysis of biological material have since been published (Table 1.1).

With an analysis time of less than two minutes per sample, PyMS is a rapid chemotaxonomic technique allowing the processing of up to 300 samples per day. Since there is no requirement for specific reagents, sample preparation is minimal. 'Curie-point' pyrolysis, developed by Simon and Giacobbo (1965), is the most commonly used method and facilitates the rapid, accurate and reproducible heating of a sample to a fixed temperature. The Curie-point of any ferromagnetic material is the temperature at which its magnetic property disappears (Wieten *et al.*, 1984). This method is fully described in chapter 2.

1.4 Applications of PyMS

Most early studies using analytical pyrolysis involved its application to relatively simple materials such as polymers (Hughes *et al.*, 1977). Improvements in instrumentation and data handling facilitated its use in an extended range of subjects including biomedical studies (Irwin & Slack, 1978)

Table 1.1
Examples of published reviews on the use of analytical pyrolysis in biological studies

Title	Authors	Date
Recent advances in pyrolysis mass spectrometry of complex biological materials	Meuzelaar *et al.*	1974
Analytical pyrolysis in biomedical studies.: A review	Irwin & Slack	1978
Analytical pyrolysis – an overview	Irwin	1979
Analytical Pyrolysis	Irwin	1982
Pyrolysis mass spectrometry of recent and fossil biomaterials	Meuzelaar *et al.*	1982
Analytical pyrolysis in clinical and pharmaceutical microbiology	Wieten *et al.*	1984
Pyrolysis mass spectrometry in bacterial systematics	Shute *et al.*	1985
Whole-organism fingerprinting	Magee	1993
Applied pyrolysis handbook	Wampler	1995

characterisation of particulate organic matter (Hemminga *et al.*, 1993; Ishiwatari *et al.*, 1995), forensic science (Hickman & Jane, 1979), soil chemistry (MacCarthy *et al.*, 1985), geochemistry (Nip *et al.*, 1985), archaeology (Pastorova et al., 1993) and the characterisation of biomarkers (Voorhees *et al.*, 1997). The use of PyMS for the analysis of complex biological systems has been extensively applied to the classification and identification of microorganisms, particularly species of medical importance (e.g., Wieten *et al.*, 1981; Morgan *et al.*, 1995; Sisson et al., 1997). Many of the principles investigated in these studies illustrate the potential use of PyMS as a chemotaxonomic tool. Weijman (1977) applied PyMS to the taxonomic discrimination of five

strains of fungi on the basis of presence or absence of chitin in their cells. Furthermore, PyMS has been successfully used in studies of organisms where taxonomic relationships were uncertain (Sutcliffe *et al.*, 1997; Lilley *et al.*, in press).

The accuracy of PyMS data has been demonstrated by congruence between identifications based on PyMS analysis and those achieved by conventional methods (e.g. Basile *et al.*, 1995). Furthermore, analysis by PyMS of some organisms has produced results comparable to DNA-based analyses. For example, PyMS data and 16s rRNA sequencing data were used comparably for the analysis of phenotypic and genotypic differences between strains of *Clostridium acetobutylicum* (Wilkinson *et al.*, 1995). The ability of PyMS to detect small genotypic changes in bacteria was also demonstrated by Goodacre and Berkeley (1990).

In some cases, the discriminatory ability of PyMS has exceeded that of biochemical methods, which may be dependent on the reactivity and / or viability of organisms. Magee *et al.* (1993) investigated pathogenicity in clinical isolates of species in the genus *Aeromonas* using traditional biochemical tests and PyMS analysis; they demonstrated that inferred pathogenicity achieved with biochemical clustering was poorer that that achieved with PyMS clustering. The discriminatory ability of PyMS has been further demonstrated in microbiology, where distinction between and within species and sub-species has been achieved (e.g., Sanglier *et al.*, 1992; Shute *et al.*, 1984; Gould *et al.*, 1991). Kay *et al.* (1994) suggested that differences detected between some organisms at the sub-species level might be interpreted as an indication of genetic drift. Freeman *et al.* (1994a) who distinguished between synthetic oligonucleotides that differed minimally in either base content or sequence further illustrated the sensitivity of this technique. PyMS analysis of crudely extracted bacterial DNA has also been shown to produce results which allow differentiation between species and sub-species (Mathers *et al.*, 1997).

1.5 Characterisation of biological samples

PyMS is not restricted to whole cell analysis and has been used to monitor the chemical composition of bacterial cell walls in response to changes in growth conditions (Boon *et al.*, 1981). In a recent study, Steijl *et al.* (1999) used PyMS to detect chemical changes in carnation stems and radish roots, which resulted from induced systemic resistance to fungal infection. Van Heemst *et al.* (1996) investigated the use of analytical pyrolysis as a tracing technique by demonstrating the algal origin of polyphenolic constituents found in dissolved organic matter and particulate organic matter sampled from the Pacific Ocean. PyMS may also be useful in studies of the mode of action of anti-microbial agents, particularly in the early screening of new compounds, and in rapid detection of the effects of antimicrobial agents on micro-organisms (Magee *et al.*, 1997a).

PyMS analysis, based on discriminant techniques with *a priori* knowledge of the data set, has been used for the purpose of confirming the identities of organisms (e.g., Magee *et al.*, 1989). The identification of 'unknown' samples using PyMS has been achieved in microbiology by the process of 'operational fingerprinting' (Meuzelaar *et al.*, 1982), which involves comparison with known reference samples included in the data set. This approach was used by Wieten *et al.* (1981) to differentiate strains of mycobacteria belonging to the 'tuberculosis complex' and other mycobacteria; a 92 % positive correlation with conventional identification tests was found. The use of PyMS for operational fingerprinting, therefore, can be seen as a rapid diagnostic method (Magee *et al.*, 1993).

Analytical pyrolysis is a technique that has been applied to a number of different biological systems and its use as a taxonomic tool has also been demonstrated, to a limited degree, in marine biology. For example, PyGC has been used for the classification of algae (Nichols *et al.*, 1968; Sprung & Wujek, 1971; Bird *et al.*, 1987). The use of PyMS as an alternative to conventional taxonomic techniques in algal systematics was reported by Russell (1995) and by Hardy *et al.* (1998) to discriminate taxa in the Phaeophyceae. However, specific investigations of the effect of environmental variability in marine algae, in either field or laboratory study, on PyMS data have not been undertaken.

1.6 Limitations of the technique

The use of PyMS for routine identification of biomaterials requires both the stability of the instrumentation and between-run reproducibility of the spectra. It is important that classifications produced by PyMS do not differ significantly from those obtained using conventional or other chemotaxonomic methods. The sensitivity of PyMS to the cultural state of organisms has been shown, in microbiological studies, to affect identification (Freeman *et al.*, 1994b). Biological variability of the sample therefore may be of significant importance in any investigation and should be minimised. Although the actual amount of sample analysed is to some extent standardised, specific sample concentrations have not been investigated. PyMS is not a typing system, as a permanent type description is not assigned to the organisms examined and it is not possible to establish a permanent fingerprint of each sample. However, this feature may be advantageous since PyMS always provides a relative form of discrimination based on analyses among the given sample set.

Assuming that all stages of sample preparation prior to analysis are standardised, several suggestions have been made with regard to the cause of poor instrumental reproducibility. These include the effect of the type of pyrolyser, contamination, instrumental drift, and the pyrolysis process itself (Irwin & Slack, 1978; Hickman & Jane, 1979; Irwin, 1982). Curie-point pyrolysis is very reproducible and the pyrolysate transfer to the ion source is also tightly controlled. The main contribution to long-term irreproducibility, therefore, is ion source ageing over periods of extended use resulting in the collection of organic debris. This alters the transmission of ions and causes mass spectral drift. Operational fingerprinting used for single batch within-run comparisons is unaffected by drift.

Manchester and Goodacre (1995) investigated short-term reproducibility of PyMS data over a four week period. They found that week to week variation was greater than day to day variation. However, variation between spectra did not deteriorate significantly after the first week and it was concluded that slight phenotypic variability (due to overnight growth of the samples) was responsible for the observed drift. Some progress on the reproducibility problem has been made by recent advances in PyMS data analysis techniques (Goodacre & Kell, 1996a).

1.7 Objectives of the present study

The principal objectives of this study were to address the potential applications and limitations of pyrolysis mass spectrometry (PyMS), as an analytical technique for marine organisms, by investigation of biological and chemical variability in macro- and micro- algae. The influence of environmental variability on the PyMS technique, within the algal groups of interest, was investigated in both natural and experimentally controlled systems using both field and laboratory studies.

Specific questions that this work aimed to address were:

1. What were the technical constraints of the PyMS method for the routine analysis of simple organic materials?
2. What was effect of variation in sample concentration on PyMS data?
3. How robust was the PyMS technique to environmentally induced variation in field population of macroalgae from the order Fucales?
4. What effect did experimentally induced variation in axenic laboratory cultures of the diatom *Skeletonema costatum* have on PyMS data?

Chapter 2

Pyrolysis Mass Spectrometry Materials and Methods

2.1 Instrumentation

2.1.1 The RAPyD-400 Pyrolysis Mass Spectrometer

The RAPyD-400 (Rapid Automated Pyrolysis Discriminator) mass spectrometer (Horizon Instruments Ltd., Heathfield, U.K.), used throughout the work reported here, was a bench-top instrument with a mass range of 12-400 daltons (Fig. 2.1). It consisted of an automated sample handling system (carousel), curie-point pyrolyser, expansion chamber, molecular beam tube, electron impact ioniser, quadrupole mass spectrometer, electron multiplier and a vacuum system (Fig. 2.2).

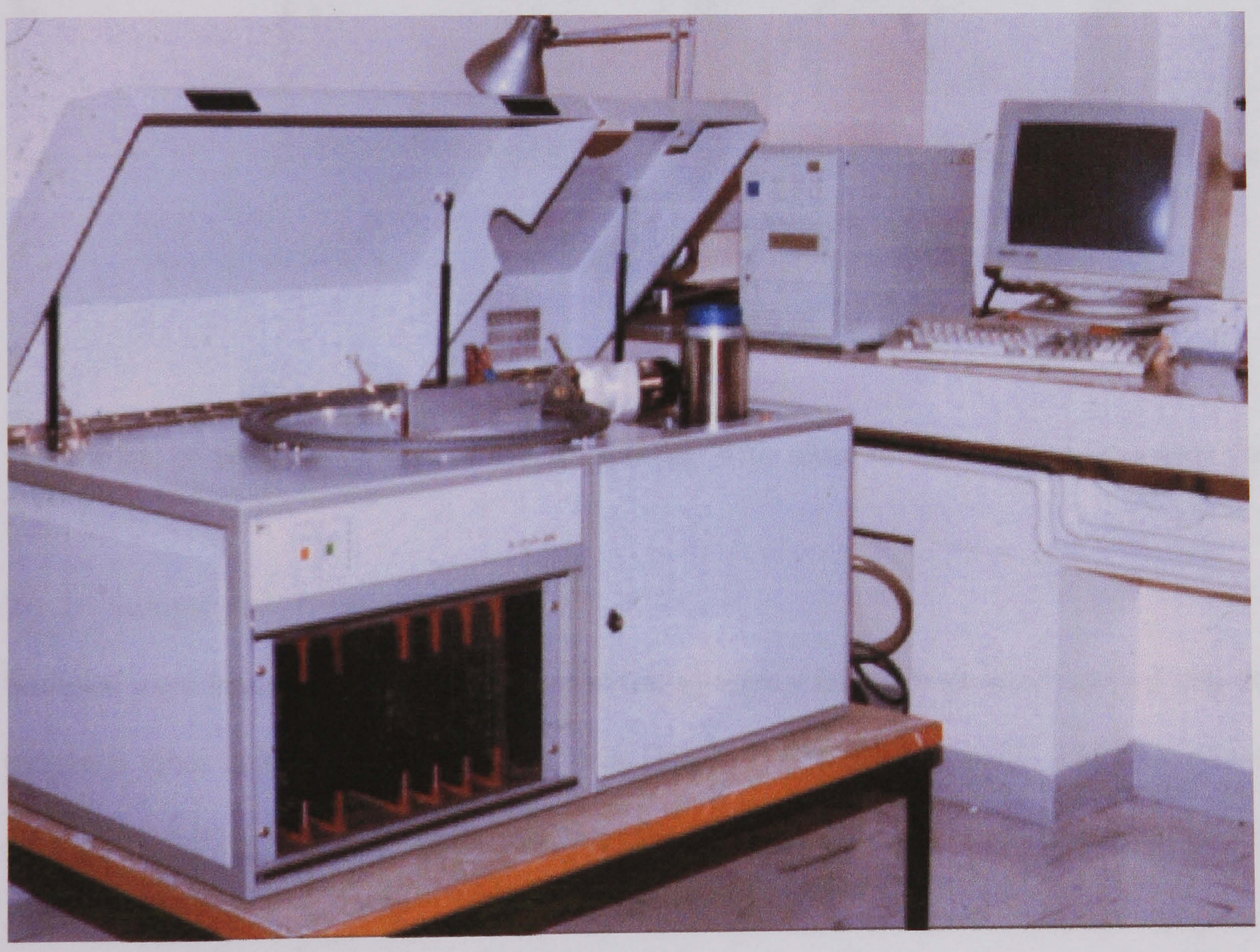


Figure 2.1
The RAPyD-400 Pyrolysis Mass Spectrometer used in this study.

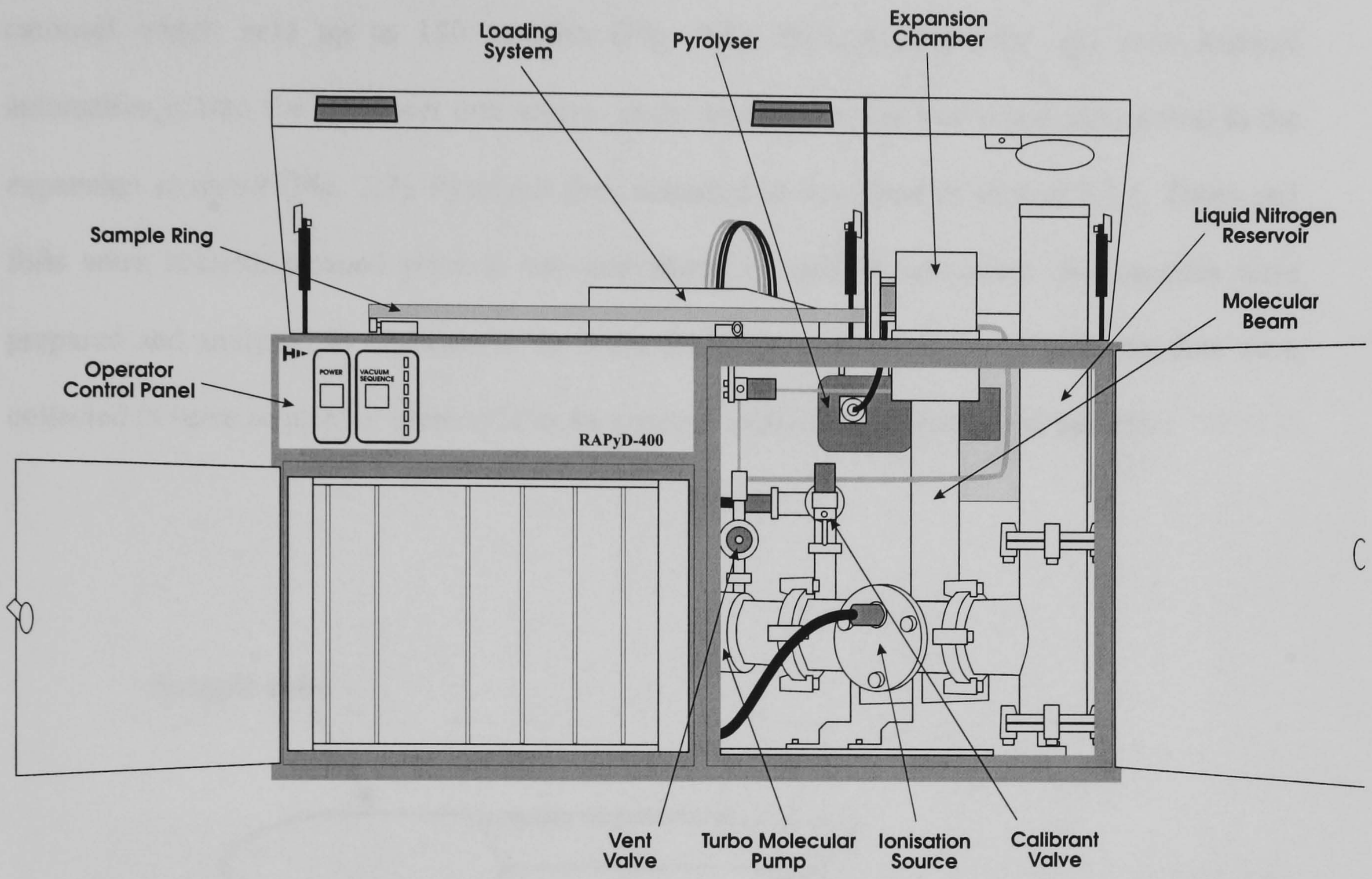


Figure 2.2
Illustration of the basic layout of the RAPyD-400 with some of the major components (from Horizon Instruments, 1988).

2.1.2 Sample preparation

Samples were loaded onto v-shaped iron: nickel pyrolysis foils, which were inserted into clean pyrolysis tubes using acetone-cleaned forceps (Fig. 2.3). Details of specific sample preparation are given in subsequent chapters. A small amount (~ 5 μg) of a solid sample was smeared evenly onto the foil using a clean disposable plastic loop (Fig. 2.4). Exact quantification of samples was not necessary as variations in sample size were corrected for during data analysis. Where samples were liquid, 5 μl aliquots were pipetted directly onto the foil. The samples were oven dried at 60° C for 10 minutes and the foils were then pushed a set distance (10 mm) into the tube

using a depth gauge (Fig. 2.5). Each tube was fitted with a viton o-ring (Horizon Instruments), to provide a seal against the inlet system, and loaded sequentially onto the PyMS auto-sampling carousel which held up to 150 samples (Fig. 2.6). Each sample tube was then inserted automatically into the pyrolyser unit where, under vacuum, it was evacuated and opened to the expansion chamber (Fig. 2.7). Pyrolysis then occurred as described in section 2.1.3. Tubes and foils were acetone cleaned prior to use and stored in airtight containers. All samples were prepared and analysed in triplicate in the order (a, b, c,) (a, b, c,) (a, b, c,) such that data were collected in three sequential identical blocks giving a total of six replicates per sample.

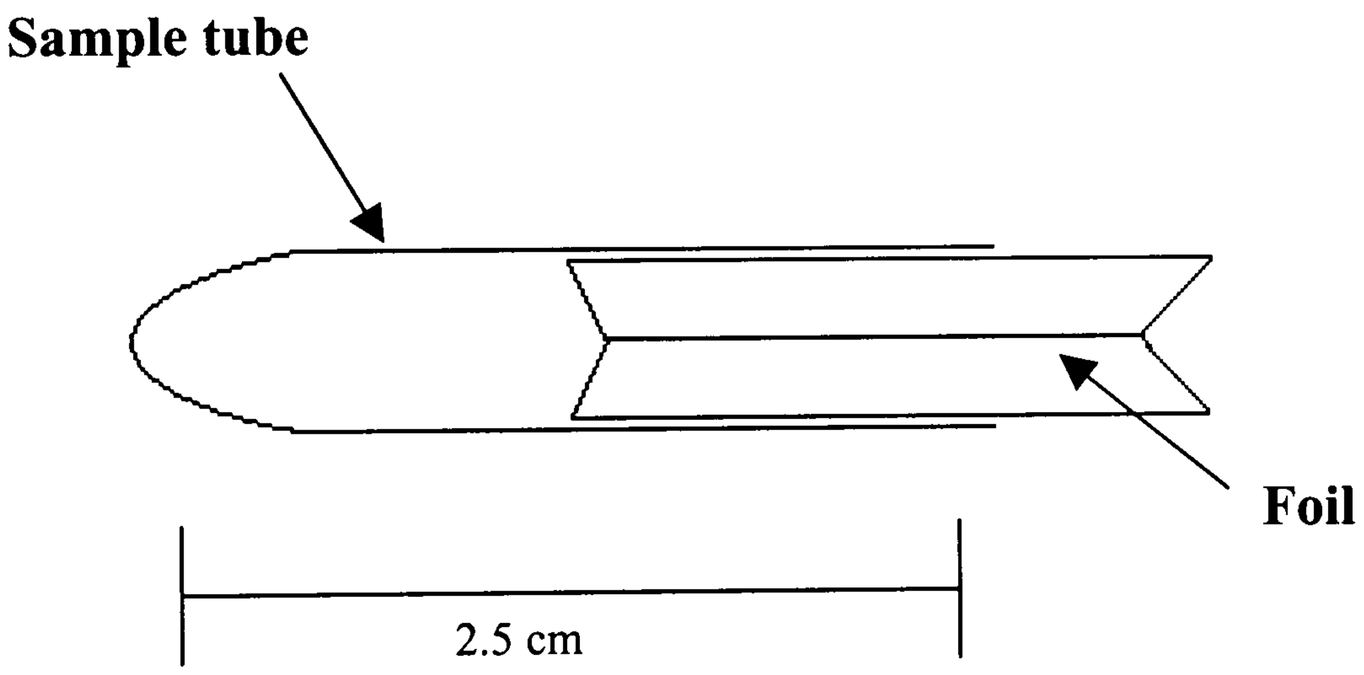


Figure 2.3
Glass pyrolysis tube with foil inserted.

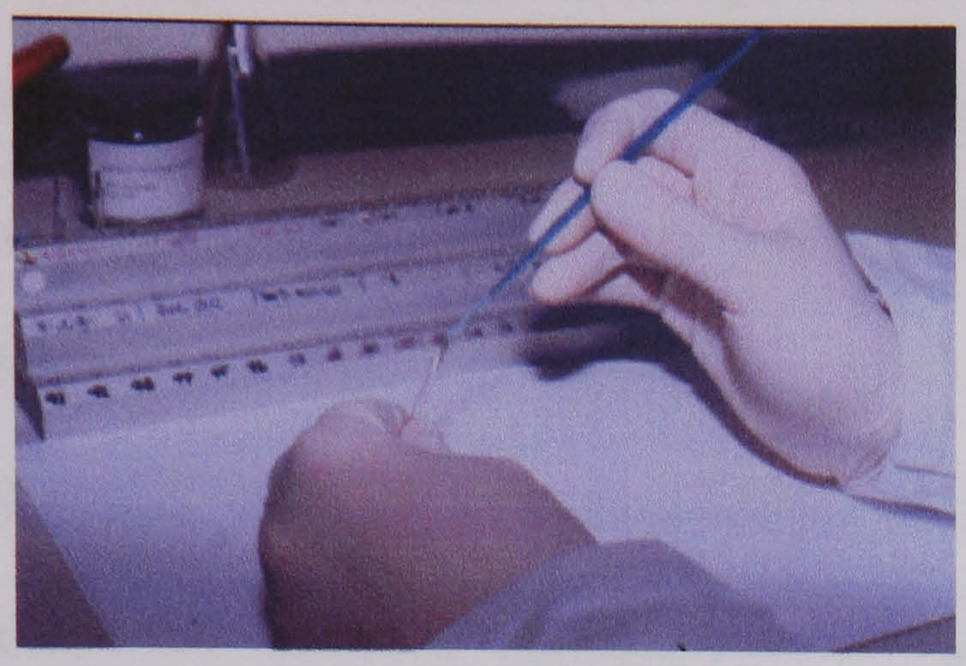


Figure 2.4
During preparation of material for analysis, a small amount of solid sample was smeared onto the foil held in the pyrolysis tube. Liquid samples were pipetted directly onto the foil.

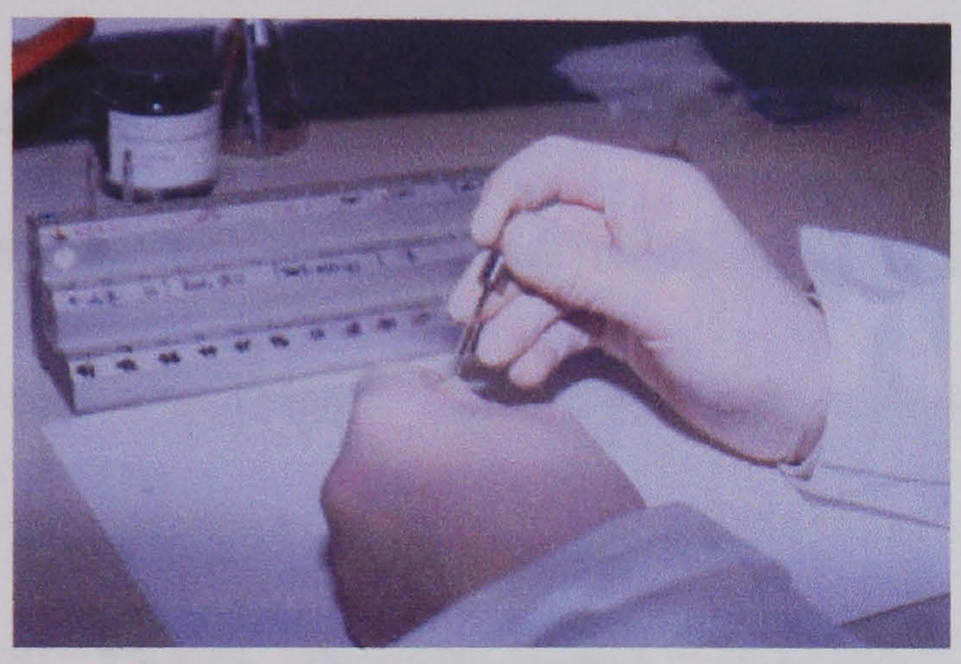


Figure 2.5
The foil was then pushed a set distance into the tube using a depth gauge.

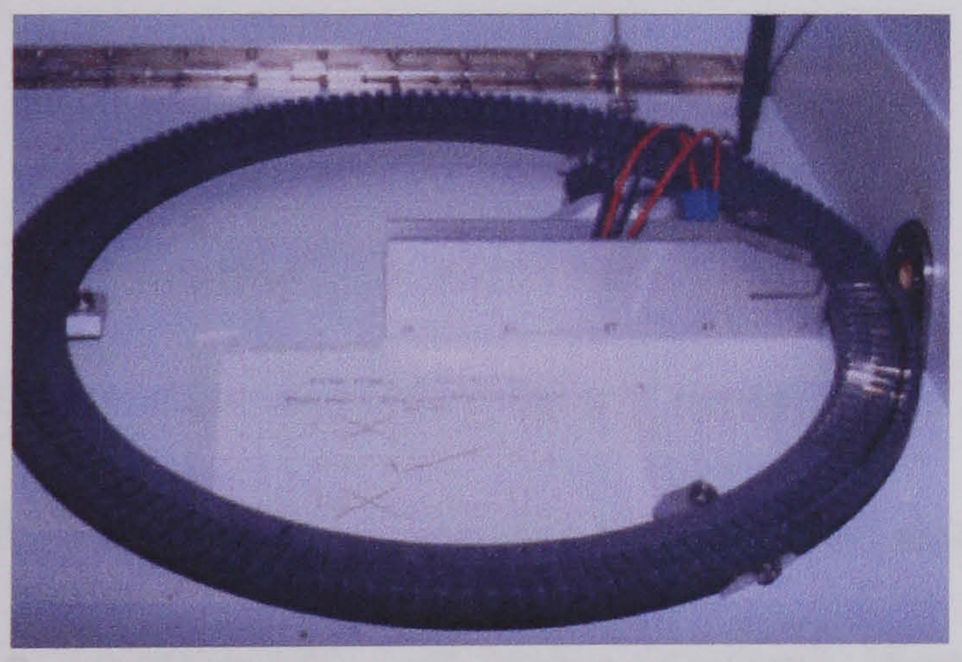


Figure 2.6
Sample tubes were loaded systematically onto the carousel prior to analysis.

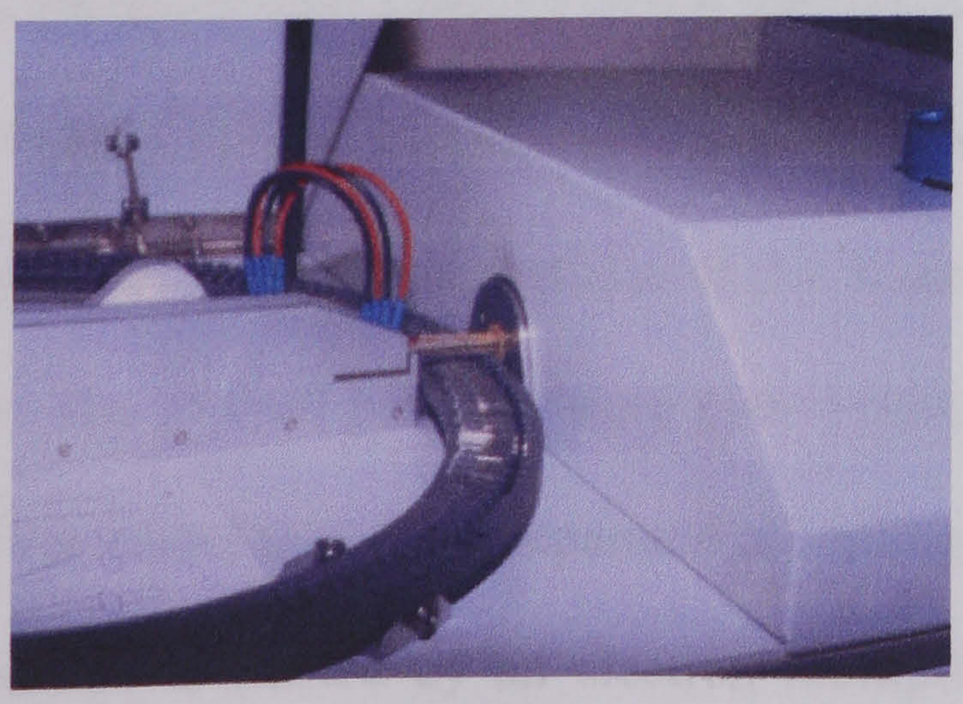


Figure 2.7
Sample tubes were loaded automatically from the carousel into the inlet system for pyrolysis.

2.1.3 Curie-point pyrolysis mass spectrometry

The Curie-point of any ferromagnetic material is the temperature at which its magnetic property disappears (Wieten *et al.*, 1984). In PyMS, the sample carrier was a magnetic foil which was heated inductively via a radiofrequency current to its Curie-point temperature in approximately 0.5s. The foil then became paramagnetic and heating ceased, but heating resumed if the temperature fell below the Curie-point such that the foil-pyrolyser system acted as a thermostatic switch (Wampler, 1995). The specific Curie-point of the foil was determined by its composition. For example, the Curie-points of cobalt, iron and nickel are 1128°C, 770°C, and 338°C respectively. Intermediate values exist for alloys of these metals. A pyrolysis temperature of 530°C (50:50 iron-nickel alloy) is commonly used for the analysis of biological materials as this results in optimum fragmentation of proteins, and polysaccharides (carbohydrates) (Windig *et al.*, 1979). Unless otherwise stated, a Curie-point of 530°C was used for all work reported in this project.

2.1.4 Sample analysis sequence

After Curie-point pyrolysis of the sample, the resultant volatile molecules (pyrolysate) passed through the expansion chamber and diffused down a molecular beam tube into the ion source of the mass spectrometer (all under vacuum). Here, the application of low energy (25eV) electron impact ionisation (EI) resulted in the formation of ions, which carried a single positive charge. Non-ionised fragments were condensed out onto a copper trap cooled by liquid nitrogen. The elimination of non-ionised particles at this stage served to prevent carryover from one sample to another. The ionised particles were then repelled by a large positive voltage into a quadrupole mass filter where they were separated according to their mass-to-charge (m/z) ratio before being detected and amplified by an electron multiplier (Goodacre & Kell, 1996b). The quadrupole consisted of four conductive rods arranged in a square configuration such that opposite pairs of

rods were connected together but with opposite polarity. This arrangement allowed only the selected mass to pass through such that the quadrupole acted as a mass filter (Meuzelaar *et al.*, 1982). After pyrolysis was completed, the ionised pyrolysate was scanned 160 times at 0.2 second intervals by the mass spectrometer. Ion counts for each mass interval were summed and presented as a pyrolysis mass spectrum in the m/z range of 51-200. This was the data collection mass range. Most biological materials produce pyrolytic degradation products, such as carbon dioxide, methane and water, the m/z ratios of which are less than 50, together with fragments with m/z greater than 200. These do not usually provide useful information for discrimination between sample groups and were ignored (Berkeley *et al.*, 1990). Sample processing, and control of operating parameters of the instrument, as well as initiation of data collection and storage were carried out under the control of an IBM-compatible computer (Aries *et al.*, 1986; Maddock & Ottley, 1995). This sequence typically occurred in 90 seconds and is summarised in Figure 2.8. Unless otherwise stated, pyrolsis conditions used throughout this work were as summarised in Table 2.1. The pyrolysis mass spectrometer was calibrated routinely (approximately monthly) throughout the period of the work reported here using the chemical standard perfluorokerosene (Sigma-Aldrich), such that the intensity of m/z 181 was one tenth of m/z 69.

Table 2.1
Pyrolysis conditions used

Temperature of inlet system	140 ° C
Temperature of expansion chamber	160 ° C
Temperature of molecular beam	180 ° C
Temperature of ion source	200 ° C
Curie-point of pyrolysis foils	530 ° C
Temperature rise time	0.5 s
Quadrupole scanning time	160 times per 0.2 s intervals
Data collection mass range	51 – 200 daltons

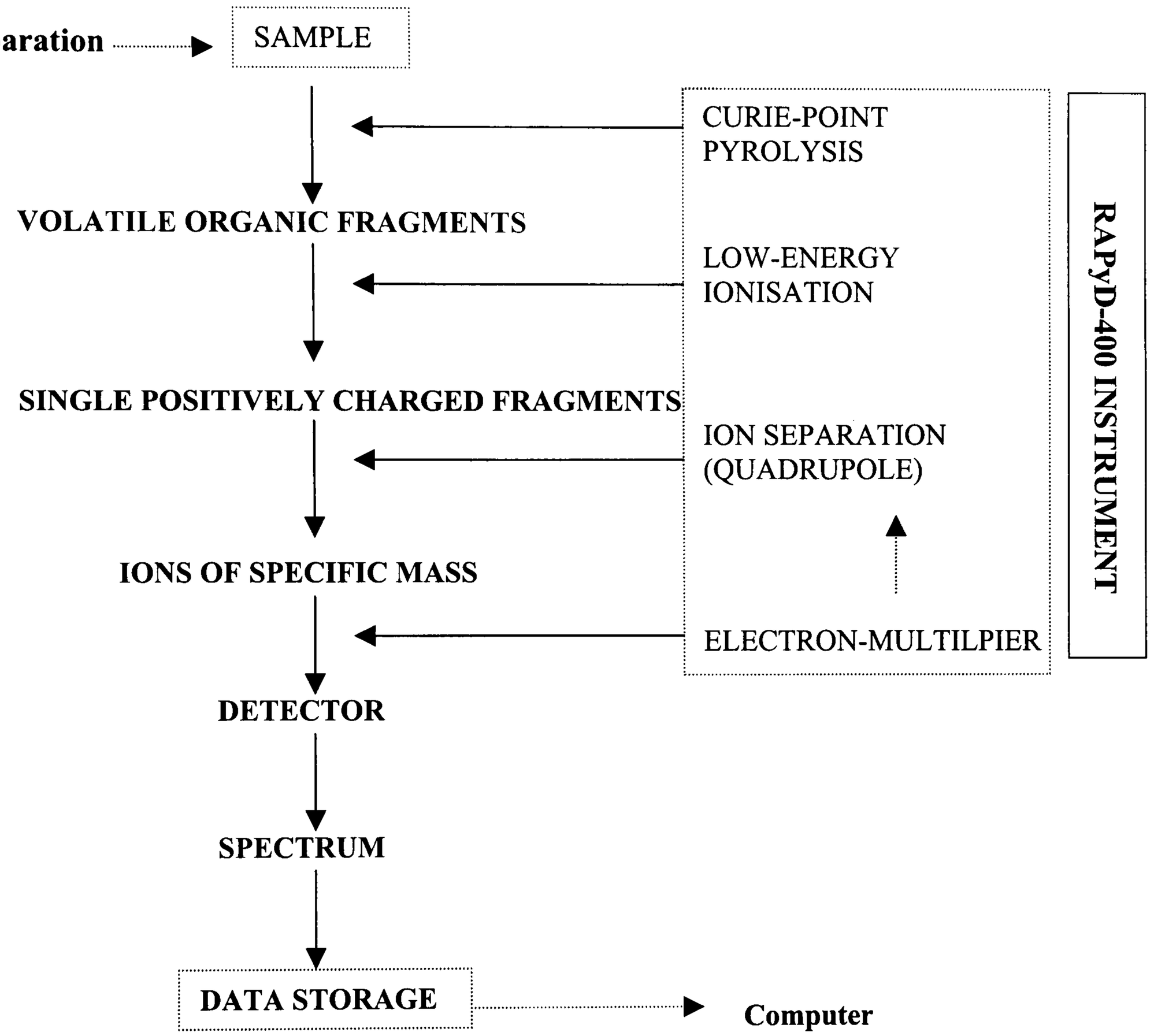


Figure 2.8
Flow diagram of the main steps in pyrolysis mass spectrometry (from Goodacre, 1994).

2.2 Data analysis

2.2.1 Multivariate data analysis

Data from PyMS were displayed as quantitative pyrolysis mass spectra showing individual masses scanned in the range 51 – 200 daltons, versus the relative ion count for each mass (mass intensity) (Fig. 2.9). The total ion count for the entire spectrum (cumulative ion count for all masses) was also recorded. PyMS data are complex and visual interpretation is unreliable due to the high degree of similarity between spectra from biological samples (Magee, 1994). Analysis of the spectral data by multivariate statistics was therefore necessary for the detection of significant differences between the spectra representing different samples. The pyrolysis data were examined initially with the GENSTAT analytical package (Nelder, 1979), using standard procedures (Gutteridge *et al.,* 1985), further details being given below. Prior to multivariate analysis of the spectra the data were first normalised to correct for variations between spectra due to differences in the amount of sample material applied to the foils. This was achieved by expressing each individual mass ion count (mass intensity) as a proportion of the total ion count (Magee, 1997) such that:

$$\text{normalised ion count} = \frac{\text{total ion count of given single mass}}{\text{total ion count of all 150 masses}} \times 100$$

Following sample normalisation, the data were ranked by a process of mass selection, termed application of 'characteristicity' in GENSTAT (Eschuis *et al.*, 1977). This procedure allowed selection of masses, which showed good reproducibility within a group of sample replicates (triplicate samples) and good discrimination between groups. Each mass in the spectrum may have multiple origins during pyrolysis, since a single mass intensity may represent the sum of contributions from several pyrolysis products, due to the formation of fragments with the same

nominal mass from different components of the sample (Windig, 1981; Magee, 1994). This results in correlation between masses within the spectrum. Therefore, differentiation of samples was achieved through the consideration of multiple masses in various combinations with one another and selection of the most characteristic masses. These may be defined as those masses which have a high ratio of between-group variation (discrimination) to within-group variation (reproducibility) (Magee, 1993, 1994). Quantitative information contained in the spectra could therefore be used for discrimination via the application of the multivariate statistical techniques principal components analysis (PCA), canonical variates analysis (CVA) and hierarchical cluster analysis (HCA).

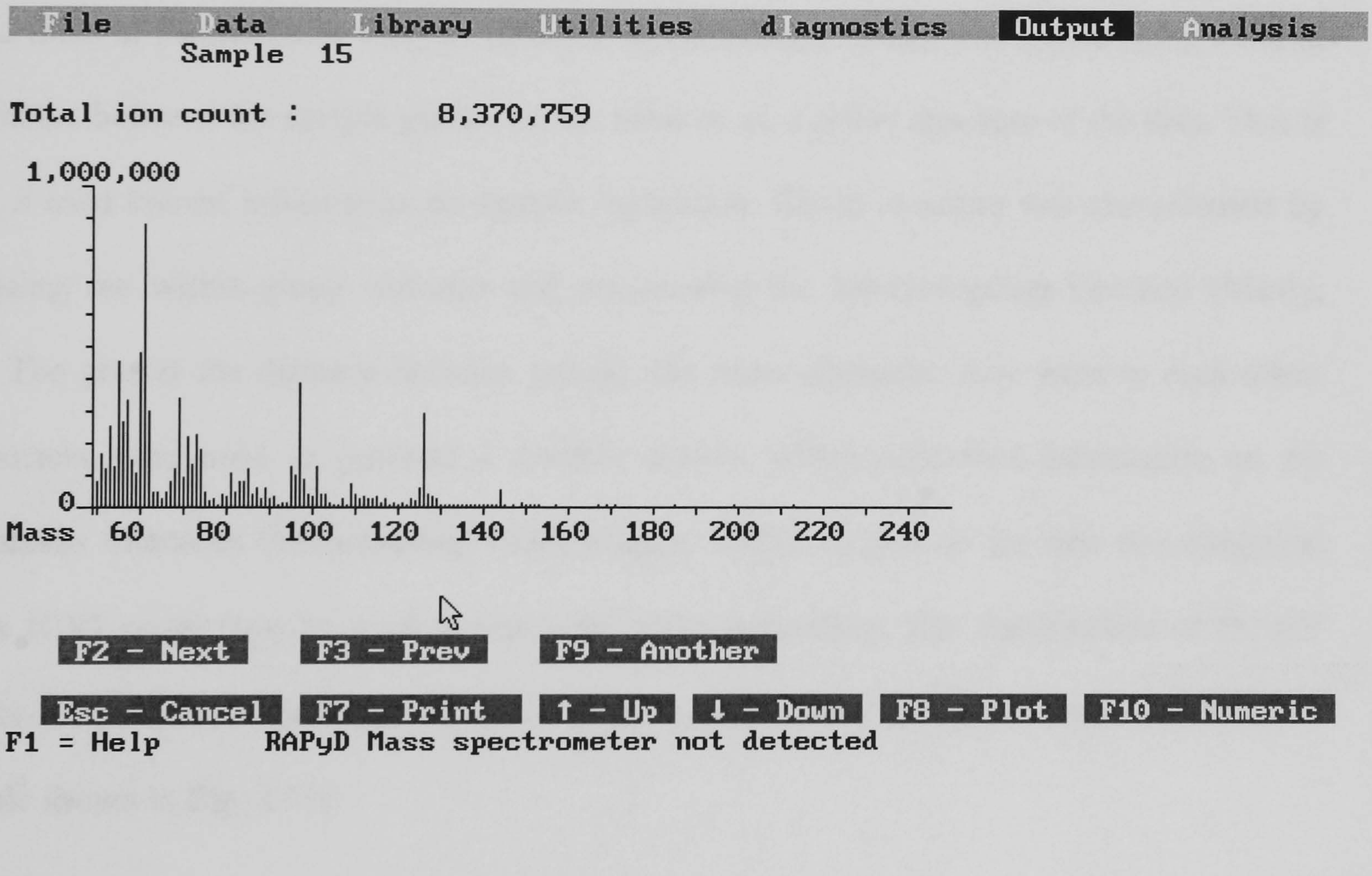


Figure 2.9
Typical pyrolysis mass spectrum output showing relative ion intensities for masses in the range 51-200 daltons.

2.2.2 Principal components analysis

PCA is essentially a data reduction technique that identifies correlations between variables (in this case masses) within the data set. PyMS data is theoretically represented in 150 dimensions,

corresponding to each of the masses scanned. PCA was useful in reducing the multiple dimensional nature of PyMS data which could then be displayed as ordination graphs illustrating natural groupings within the data in two dimensions. The total variance within the data could be represented as a plot of the first two principal components (PC), which account for most of the variation (>85 %) within the data set (Gutteridge *et al.*, 1985) (Fig. 2.10). The values of the principal components were derived through extraction of the eigenvalues of the variance matrix (Goodfellow *et al.*, 1997). Sample outliers could then be identified from the PC plots and were removed from subsequent analyses (Shute *et al.*, 1984).

2.2.3 Canonical variates analysis

CVA, a form of discriminant analysis (MacFie & Gutteridge, 1982), was applied after PCA to discriminate between the sample groups on the basis of an *a priori* structure of the data. That is to say, it used known information on sample replication. Group structure was characterised by minimising the within-group variance and maximising the between-group variance (Manly, 1994). The greater the distance between groups, the more dissimilar they were to each other. Eigenvectors were used to generate a distance matrix, which contained information on the Mahalanobis distances (Mahalanobis, 1936; Magee, 1993). A plot of the first two canonical variates (CV) could then be used to represent group separation. The combination of PC-CV analyses of PyMS data was displayed as two or three dimensional ordination plots in GENSTAT (example shown in Fig. 2.11).

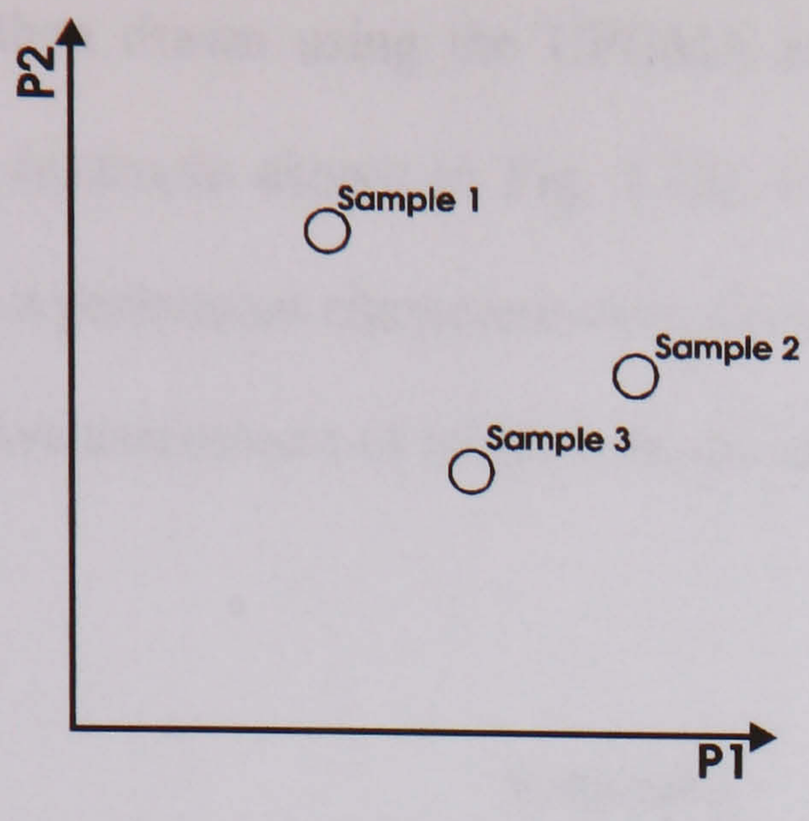


Figure 2.10
Example of ordination plot showing the first two principal components (P1, P2) generated from analysis of PyMS data.

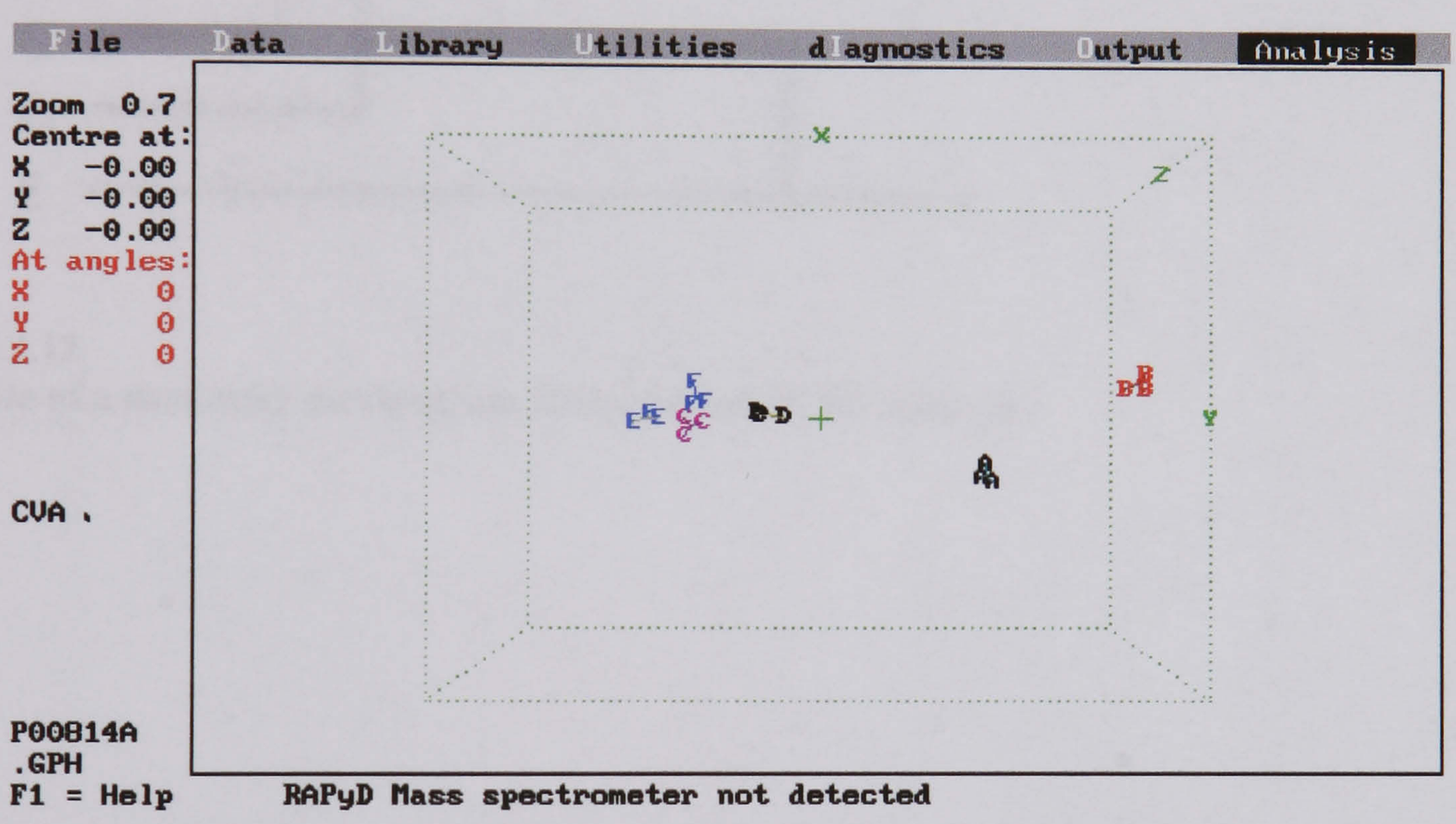


Figure 2.11
Example of 3D PC-CV GENSTAT plot following analysis of PyMS data.

2.2.4 Hierarchical cluster analysis

Following PCCV analysis of PyMS data, hierarchical cluster analysis (HCA) was used to transform the Mahalanobis distance matrix into a percentage similarity values using Gower's similarity coefficient S_G (Gower, 1971). Dendrograms showing hierarchical group clustering

were then drawn using the UPGMA average linkage clustering algorithm (Sneath & Sokal, 1973) (example shown in Fig. 2.12). Unlike conventional typing techniques, PyMS does not assign a permanent characterisation since discrimination applied within each batch only and was a relative assessment of relatedness (Sisson *et al.*, 1993).

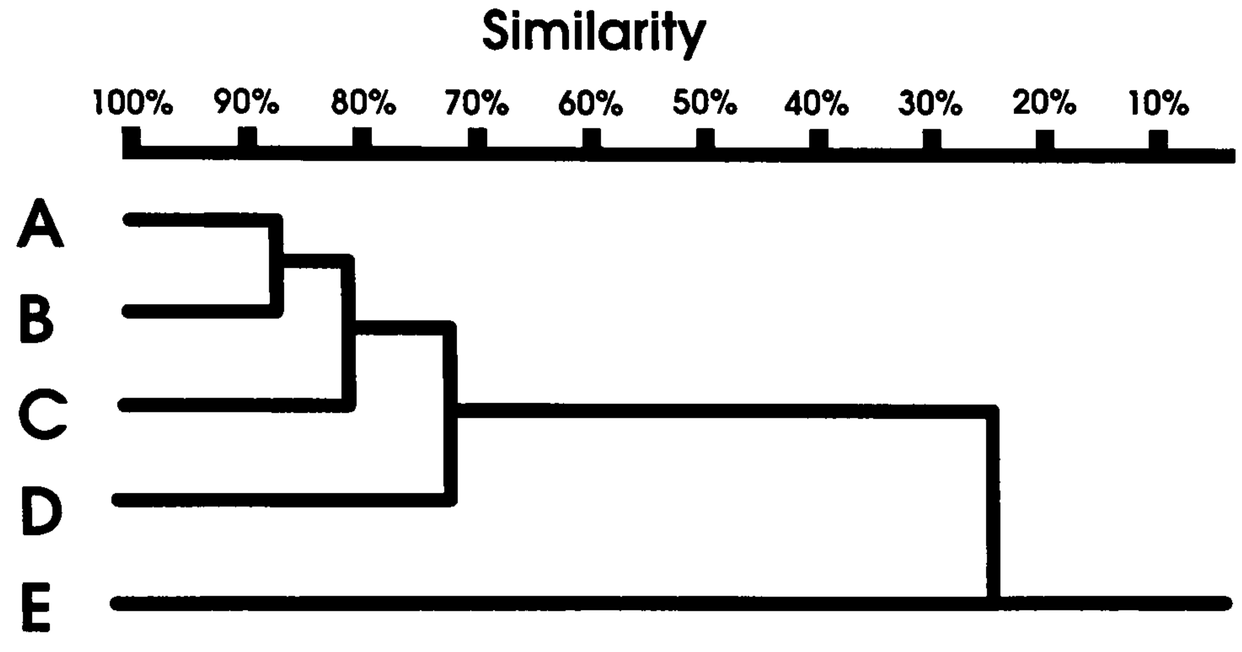


Figure 2.12
Example of a similarity dendrogram derived from PyMS analysis.

Chapter 3

Development and Appraisal of the PyMS Technique

3.1 Introduction

PyMS has been shown to be a rapid, universally applicable and highly discriminatory technique (e.g. Goodacre and Kell, 1996; Goodfellow *et al.*, 1997); it has proved, for example, to be particularly useful in identification, or the assignation of a particular organism to a specific taxonomic group, where other reproducible discriminatory tests available are limited. Magee *et al.* (1989) used PyMS to discriminate taxa in the Fusobacteria, which present classification and identification problems due to their lack of reactivity in conventional tests. The sensitivity of PyMS has also been shown by the detection of small genotypic changes in bacteria. For example, Goodacre and Berkeley (1990) discriminated between four closely related strains of *Escherichia coli*, which differed by the presence or absence of a single antibiotic resistance plasmid. PyMS was also used to successfully characterise micro-organisms by gram-type on the basis of their fatty-acid distributions (Basile *et al.*, 1995).

However, lack of reproducibility has been identified as a limiting factor in the use of PyMS as an analytical tool (Eschuis *et al.*, 1977; Irwin & Slack, 1978; Hickman & Jane, 1979; Shute *et al.*, 1988). If PyMS is to be used on a routine basis, which would involve the comparison of a mass pyrogram of a particular sample with a library collection of mass pyrograms, then the reproducibility of the system becomes an important factor. The appearance of the pyrogram reflects the composition of the sample and the pyrolysis conditions, however some peaks in the pyrogram may show large variations within the same class of samples. This high level of inner variance has been attributed to variations in the sample itself (i.e. biological variability or phenotypic drift), or in the sample preparation procedure prior to analysis (Eschuis *et al.*, 1977). For the successful application of PyMS for identification and discrimination of biological samples, it is important that spectral differences caused by this inner variance be smaller than the

differences between different organisms. The effect of biological variation between similar samples can be minimised, within one sample run, by pyrolysing at least three representatives of each sample, and more than one sample from each organism (Freeman *et al.*, 1994b). Additionally, growth conditions and sample preparation should be standardised as far as possible (Voorhees *et al.*, 1988).

Reproducibility of the physical conditions of pyrolysis mass spectrometers over time is another area of variability in PyMS analyses. Hickman and Jane (1979) investigated various parameters of the pyrolysis system itself as sources of error in the reproducibility of PyMS data. They identified the pyrolysis process, and not the mass spectrometric detection, as the cause of variation between data collected for a set of forensic samples over a six-week period. However, a study carried out by Windig *et al.* (1979) on the effects of sample preparation and pyrolysis conditions showed that short-term irreproducibility could largely be overcome by using standard and well-defined instrument parameters. Short-term reproducibility of PyMS data was also investigated in a study by Manchester *et al.* (1995), who looked at the effect of culture-age of five strains within the genus *Carnobacterium* on PyMS classification and the reproducibility of the groupings produced over a four-week period. They observed sustainable separation of the five type-strains over the experimental period and representatives were consistently recovered in species-specific groups. A number of studies have been carried out to determine the long-term stability and inter-batch reproducibility of PyMS classifications (e.g. Shute *et al.*, 1988). The lack of long-term reproducibility of PyMS data has been attributed to mass spectral drift, that is, to changes in ion transmissivity due to contamination and ageing of the ion-source of the mass spectrometer (Windig *et al.*, 1979; 1980; Goodacre & Kell, 1996b). Recent developments in data analysis techniques have focussed on the use of artificial neural networks and other multivariate calibration models to relate mass spectra to various biological features (Chun *et al.*,

1993; Freeman *et al.*, 1994c). Subsequently, some success has been achieved in using neural networks to facilitate identification across pyrolysis mass spectrometric batches (Goodacre & Kell, 1996a; Goodacre *et al.*, 1997).

Sample size has also been found to influence the short-term reproducibility of pyrolysis mass spectra. A study by Windig *et al.* (1979) showed that pyrolysis mass spectra obtained from 1 μg quantities of bovine serum albumin (BSA) and glycogen clearly differed from the pyrolysis mass spectra obtained from larger samples (5–20 μg) of the same substances. This difference was attributed to the relatively large contribution of the ambient spectrum of small samples, due to some residual contamination of the ion source and expansion chamber.

The aims of the current study were to determine the effect of sample concentration on PyMS spectral data and the sensitivity of the technique at various concentrations. A series of chemicals, representative of simple organic materials, were used for this experiment. The effect of any instrument drift on the reproducibility of PyMS outputs over time were also investigated.

3.2 Materials and Methods

Standard pyrolysis conditions and instrumentation were used as previously described in chapter 2.

3.2.1 Amino acid solutions and concentrations

Four amino acids, namely lysine, phenylalanine, alanine, glycine, and a biopolymer, glycogen, (Sigma-Aldrich), were prepared in solutions at six different concentrations (Table 3.1). Six microlitres of each solution at each concentration were analysed in triplicate as described in section 2.1, on days 1, 2, 7, 14, 21 and 28 of a 28-day experimental period. Between analyses all solutions ere stored at –20 °C to minimise any changes in their chemical nature. The same stock

solution of each amino acid and each concentration was used throughout the experimental period.

Table 3.1.
Weight (μg) and concentration (μM) of each of 5 amino acid solutions applied to pyrolysis foils in 6 μl of solution.

% conc. of solution	Conc. (μM)	Weight (μg) in 6μl of solution				
		Glycogen	Lysine	Phenylalanine	Alanine	Glycine
100	**60**	60	65.7	59.5	32.1	27
50	**30**	30	32.9	29.7	16.1	13.5
20	**12**	12	13.1	11.9	6.4	5.4
10	**6**	6	6.6	5.9	3.2	2.7
5	**3**	3	3.3	3.0	1.6	1.4
1	**0.6**	0.6	0.66	0.59	0.32	0.27

3.2.2 Data analysis

All data were pre-processed as described in section 2.2.1. The spectra obtained for high concentrations were compared with those obtained for lower concentrations on both the same sample day and over the entire experimental period. The occurrence of any instrument drift over time was examined by comparing the PyMS spectral data collected for identical sample solutions on different days. Principal components (PCA) and canonical variates (CVA) analyses were also applied to all spectra. ANOSIM analyses were carried out using the PRIMER v4.0 software package (Plymouth Marine Laboratory, NERC, 1994).

3.3 Results

3.3.1 Effect of sample concentration

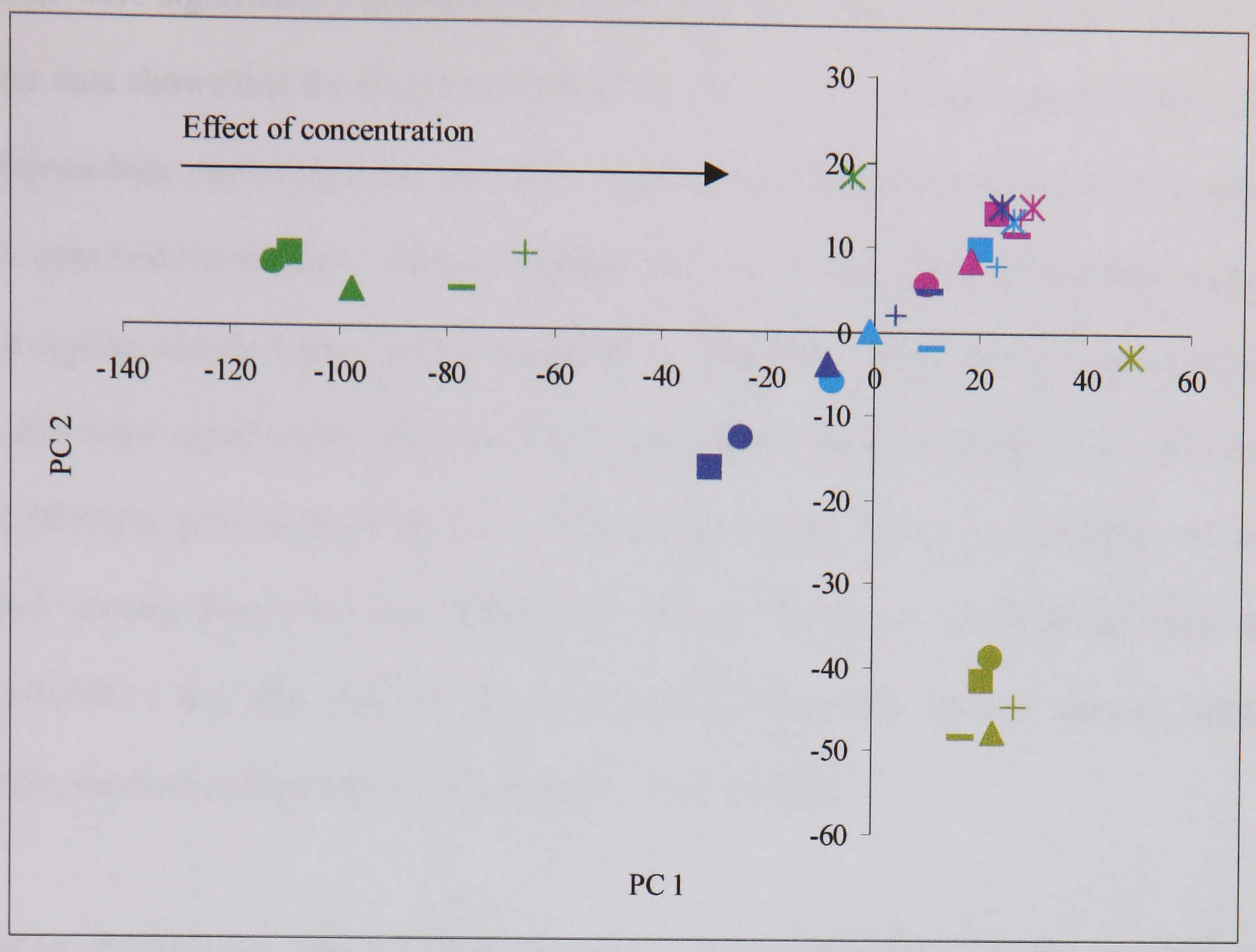
There were overall differences in pyrolysis mass spectra among all solutions (ANOSIM, R= 0.87, $p<0.0001$) and all concentrations (ANOSIM, R=0.37, $p<0.0001$) (Fig. 3.1, Table 3.2). ANOSIM pairwise tests showed that each concentration, within each solution, was different

from all others except for the one closest to it (Table 3.3). That is to say that concentrations 60μM and 30μM, 30μM and 12μM, 12μM and 6μM, and 6μM and 3μM were not significantly different from each other in all solutions. Distinction between sample groups at the 0.6μM concentration was most difficult to observe (Fig. 3.1). Glycogen, a biopolymer, was clearly separated from the four amino acid sample groups at all concentrations but the amino acids glycine and alanine, which differ in their structure by virtue of one methyl group only, notably formed a close cluster in all cases (Fig. 3.1).

Table 3.2
One-way ANOSIM results comparing all concentrations of all solutions analysed on day 1.

Factor	Global R	P
Alanine all concentrations	0.37	< 0.0003
Glycine all concentrations	0.007	> 0.05 (= 0.074)
Glycogen all concentrations	0.65	< 0.00002
Lysine all concentrations	0.57	< 0.00002
Phenylalanine all concentrations	0.22	< 0.002

Total ion count was also observed to vary with sample concentration. A significant positive correlation between concentration and total ion count was recorded for glycogen ($r=0.96$; $p<0.0001$), alanine ($r=0.72$; $p<0.001$), phenylalanine ($r=0.54$; $p<0.05$) and lysine ($r=0.78$; $p<0.0001$) (Fig. 3.2 a-d). No significant correlation was recorded for glycine ($r = 0.38$; $p>0.05$) (Fig. 3.2 e). Similar correlations between total ion count and concentration of solution were observed for all other days.



Glycogen: 60 µM •, 50 % ■, 20 % ▲, 10 % –, 5 % +, 1 % *

Phenylalanine: 60 µM •, 50 % ■, 20 % ▲, 10 % –, 5 % +, 1 % *

Alanine: 60 µM •, 50 % ■, 20 % ▲, 10 % –, 5 % +, 1 % *

Lysine: 60 µM •, 50 % ■, 20 % ▲, 10 % –, 5 % +, 1 % *

Glycine: 60 µM •, 50 % ■, 20 % ▲, 10 % –, 5 % +, 1 % *

Figure 3.1
Principal component 1 (PC1) plotted against principal component 2 (PC2) for PyMS data (means, n = 3) of solutions of 4 amino acids and glycogen at all concentrations from day 1.

3.3.2 Effect of time on sample discrimination

The effect of instrument drift over time on sample discrimination is illustrated using data for all concentrations of glycogen (Fig. 3.3). Samples of the same concentration were seen to cluster together in the PC-CV plot, irrespective of the day on which they were analysed. However, the spectra obtained for concentrations were significantly different among days (Table 3.4a) and

concentrations were significantly different from each other on all days (Table 3.4b). The PC-CV plot for these data shows that the 60 μM and 0.6 μM solutions were most distinct from each other and that intermediate concentrations were seen to cluster uniformly between the two. Similar results were obtained for all other solutions. PyMS data obtained for the five solutions analysed showed that similar sample types clustered together, independent of the day on which they were processed, and were significantly different from each other at both 60μM ($R=0.94$, $p<0.0001$) and 0.6μM ($R=0.86$, $p<0.0001$) (Figs. 3.4 – 3.5, Table 3.5a-b). There was evidence of some machine drift among these data and there were overall differences between analysis days ($R=0.72$, $p<0.0001$) but this did not result in mis-identification of any sample solution throughout the experimental period as shown by the PC-CV plots.

Table 3.3
Pairwise test results from two-way ANOSIM among all concentrations for all solutions analysed on day 1.

Concentration combinations	Global R	P
(60 μM, 30 μM)	-0.0001	= 0.497
(60 μM, 12 μM)	0.37	< 0.009
(60 μM, 6 μM)	0.62	< 0.0001
(60 μM, 3 μM)	0.59	< 0.001
(60 μM, 0.6 μM)	0.64	< 0.0001
(30 μM, 12 μM)	-0.026	= 0.56
(30 μM, 6 μM)	0.37	< 0.009
(30 μM, 3 μM)	0.58	< 0.0001
(30 μM, 0.6 μM)	0.69	< 0.0001
(12 μM, 6 μM)	0.15	= 0.13
(12 μM, 3 μM)	0.29	< 0.015
(12 μM, 0.6 μM)	0.73	< 0.0001
(6 μM, 3 μM)	-0.052	= 0.67
(6 μM, 0.6 μM)	0.51	< 0.0001
(3 μM, 0.6 μM)	0.40	< 0.004

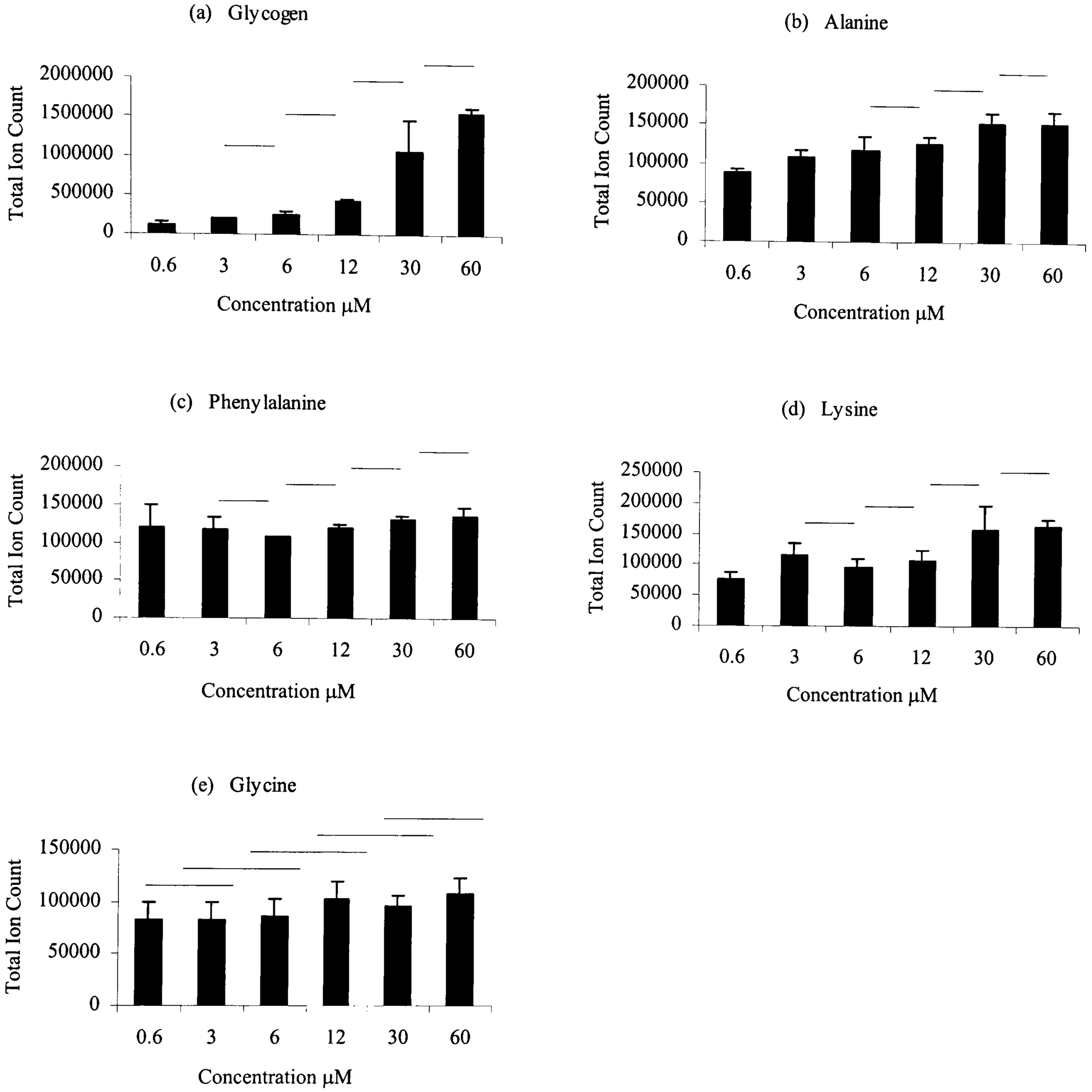


Figure 3.2

Plots of mean (n = 3) ion count against concentration for (a) glycogen, (b) alanine, (c) phenylalanine, (d) lysine, (e) glycine, analysed on day 1. Overlapping bars indicate no significant difference between concentration groups. Error bars show standard deviations.

Table 3.4 (a – b)
One-way ANOSIM results for glycogen: (a) among concentrations for days 1 – 28, and (b) among days for all concentrations.

(a) Among days, within concentrations

Factor	Global R	p
60 µM	0.19	< 0.003
30 µM	0.061	< 0.04
12 µM	0.15	< 0.001
6 µM	0.32	< 0.001
3 µM	0.54	< 0.00002
0.6 µM	0.40	< 0.0001

(b) Among concentrations, within days.

Factor	Global R	p
Day 1	0.77	< 0.00002
Day 2	0.71	< 0.00002
Day 7	0.87	< 0.00002
Day 14	0.56	< 0.00002
Day 21	0.81	< 0.00002
Day 28	0.69	< 0.00002

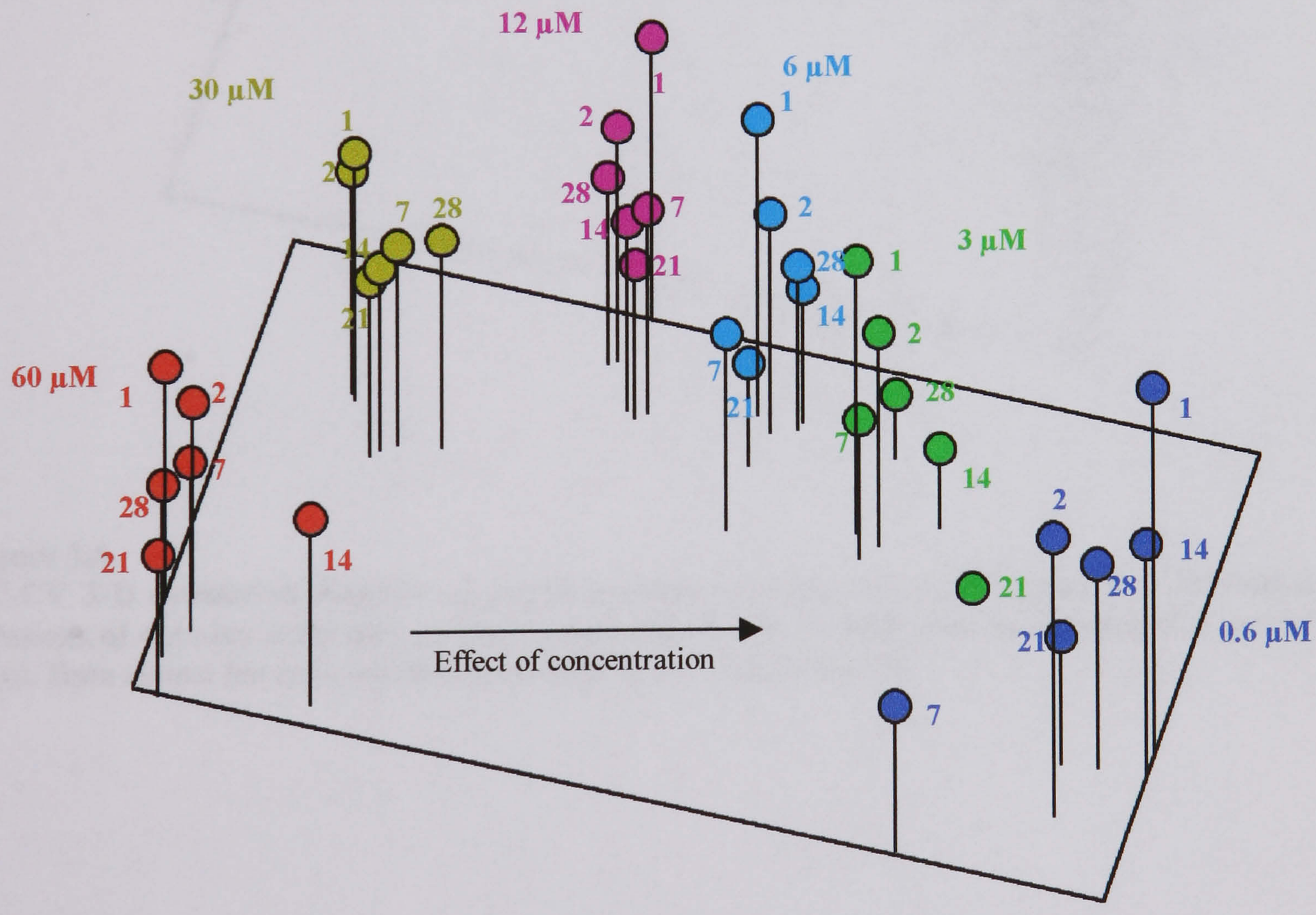


Figure 3.3
PC-CV 3-D ordination diagram of pyrolysis mass spectrometry data (means, n = 3)obtained for different concentrations of glycogen (60 µM, 30 µM, 12 µM, 6 µM, 3 µM and 0.6µM) over an experimental period of 28 days. Data shown from days 1, 2, 7, 14, 21 and 28.

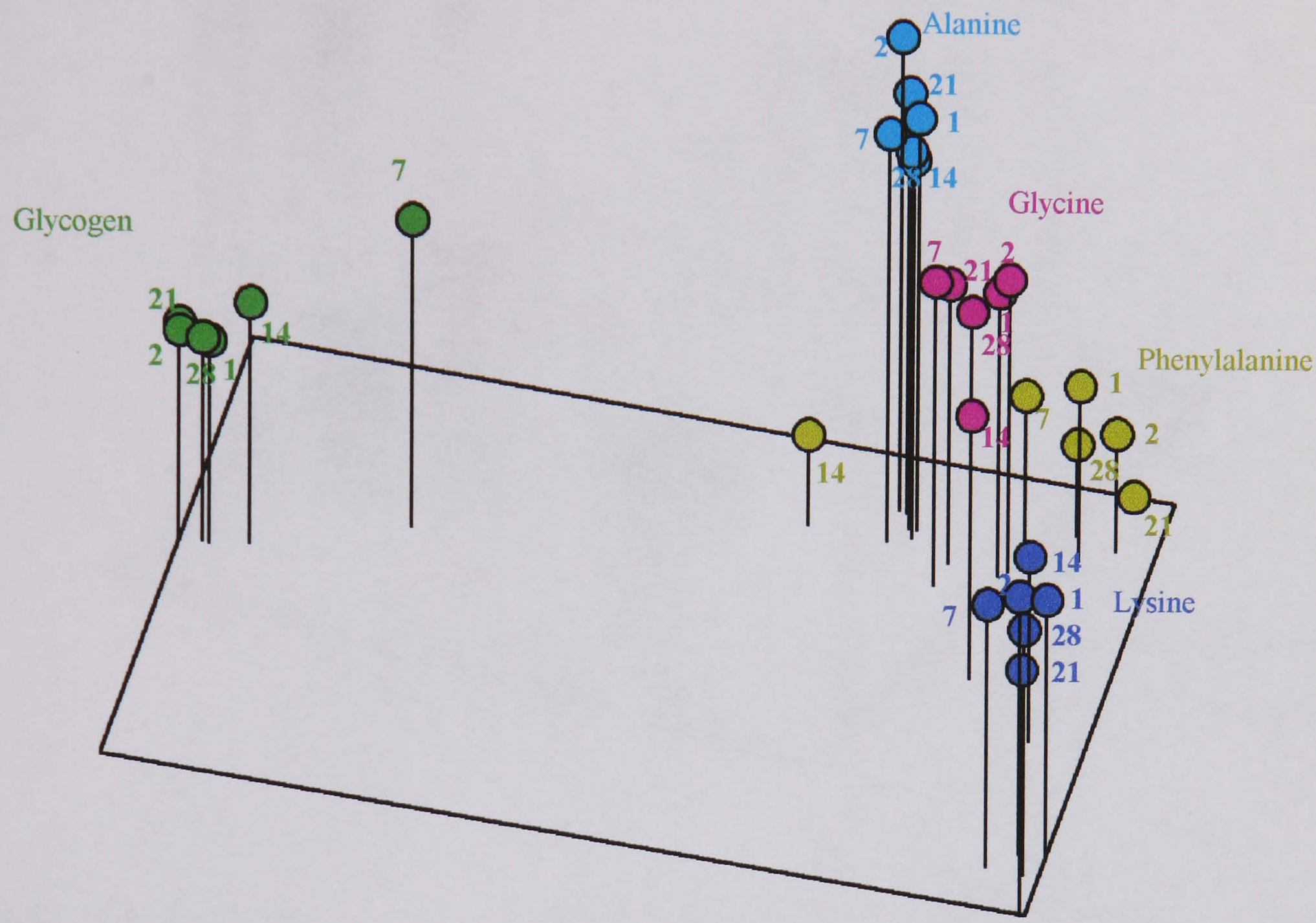


Figure 3.4
PC-CV 3-D ordination diagram of pyrolysis mass spectrometry data (means, n = 3) obtained for solutions of 4 amino acids and glycogen (concentration at 60 μM) over an experimental period of 28 days. Data shown for each solution from days 1, 2, 7, 14, 21 and 28.

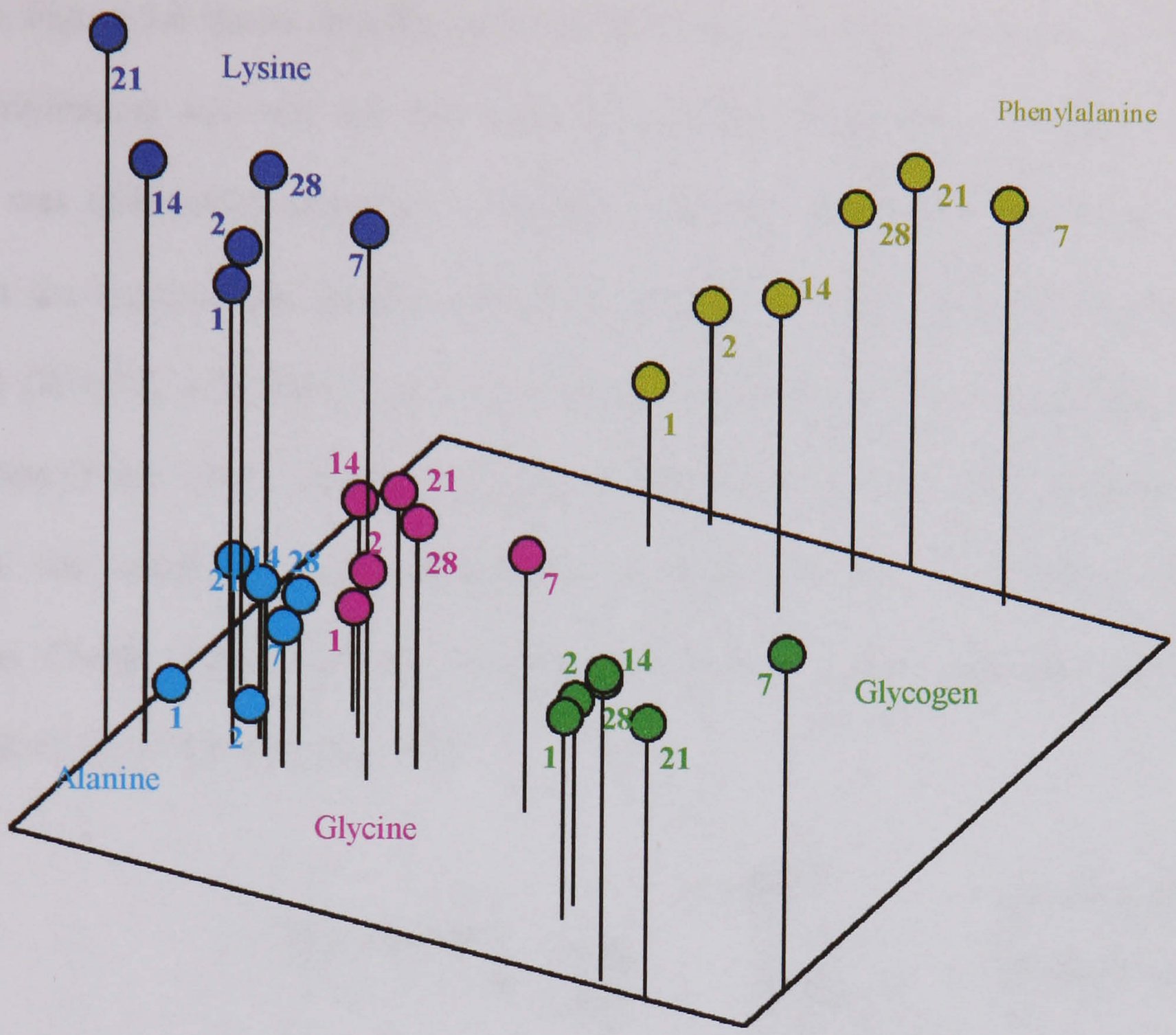


Figure 3.5
PC-CV 3-D ordination diagram of pyrolysis mass spectrometry data (means, n = 3) obtained for solutions of 4 amino acid and glycogen (concentration at 0.6 μM) over an experimental period of 28 days. Data shown for each solution from days 1, 2, 7, 14, 21 and 28.

Table 3.5 (a-b)
One-way ANOSIM results for solutions at concentrations: (a) 60 μM, and (b) 0.6 μM among days.

(a) 60 μM

Factor	Global R	p
Alanine conc.	0.23	< 0.0025
Glycine conc.	0.36	< 0.002
Lysine conc.	0.34	< 0.0001
Phenylalanine conc.	0.43	< 0.0007
Glycogen conc.	0.18	< 0.004

(b) 0.6 μM

Factor	Global R	p
Alanine conc.	0.55	< 0.00002
Glycine conc.	0.10	< 0.00002
Lysine conc.	0.84	< 0.00002
Phenylalanine conc.	0.81	< 0.00002
Glycogen conc.	0.85	< 0.00002

Glycogen and glycine were chemically the most dissimilar to each other among the solutions analysed here. Figure 3.6 shows data for these two solutions, at all concentrations, for days 1 and 2 only. Discrimination between the two different solutions could be seen from the PC-CV analysis and was statistically significant (ANOSIM, $R=0.83$, $p<0.0001$). However, ANOSIM performed on the spectral data showed that there were overall differences among the spectra between days ($R=0.02$, $p<0.0001$). All concentrations analysed were also significantly different among solutions (Table 3.6a). Analysis of glycogen and glycine between days 1 and 28 only also showed that the solutions were significantly different ($R=0.83$, $p<0.0001$) among all concentrations (Table 3.6b) but that spectral drift between days was also of statistical significance ($R=0.17$, $p<0.0001$) (Fig. 3.7).

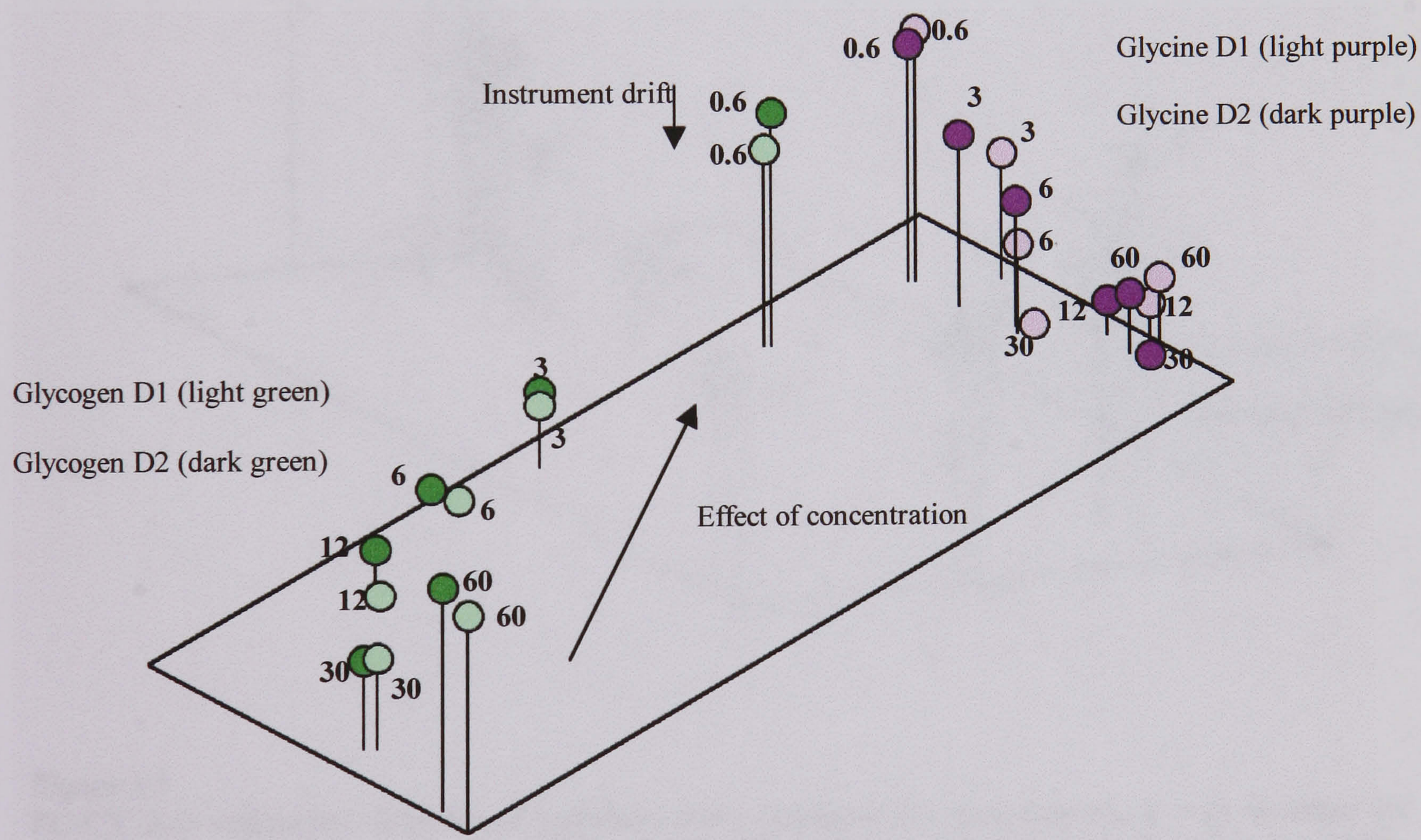


Figure 3.6
PC-CV 3-D ordination diagram of pyrolysis mass spectrometry data (means, n = 3) obtained for glycogen and glycine solutions (60 μM, 30 μM, 12 μM, 6 μM, 3 μM and 0.6μM) from days 1 and 2.

Table 3.6 (a-b)
One-way ANOSIM results for glycine and glycogen among all concentrations compared for: (a) days 1 and 2, and (b) days 1 and 28.

(a) Days 1 and 2

Factor	Global R	p
Glycine Day 1	0.088	< 0.04
Glycogen Day 1	0.76	< 0.00002
Glycine Day 2	0.23	< 0.003
Glycogen Day 2	0.72	< 0.00002

(b) Days 1 and 28

Factor	Global R	p
Glycine Day 1	0.057	< 0.05
Glycogen Day1	0.75	< 0.00002
Glycine Day 28	0.21	< 0.006
Glycogen Day28	0.69	< 0.00002

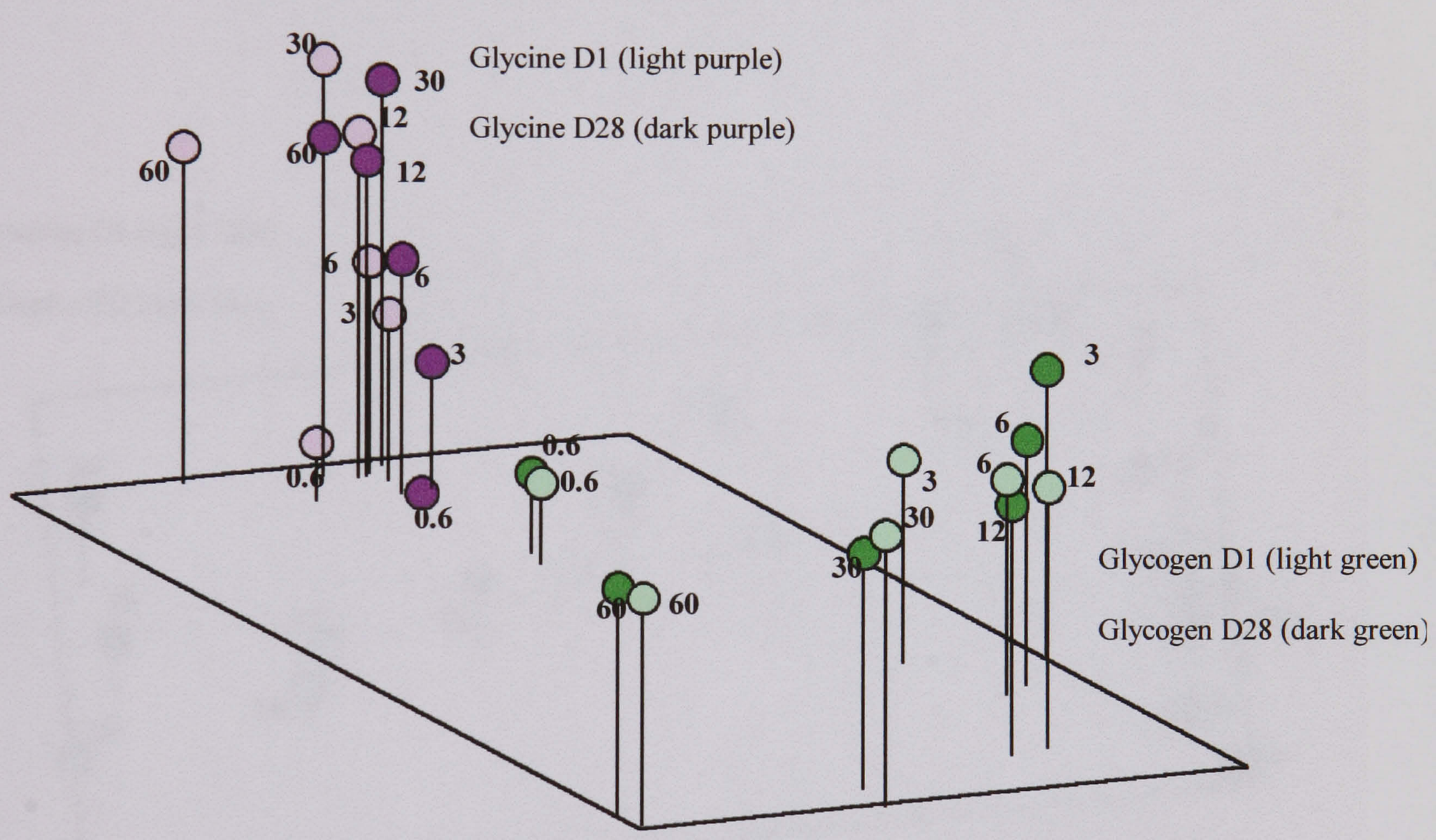


Figure 3.7
PC-CV 3-D ordination diagram of pyrolysis mass spectrometry data (means, n = 3) obtained for glycogen and glycine solutions (60 μM, 30 μM, 12 μM, 6 μM, 3 μM and 0.6μM) from days 1 and 28.

3.3.1 Sensitivity of the technique

The sensitivity of the PyMS technique was highlighted by the PC-CV discrimination of all groups at the 0.6μM concentration (Fig. 3.5). In all PC-CV analyses the separation between

alanine and glycine was smaller than that observed for all other samples. However, these groups were separated into two significantly different clusters upon PC-CV analyses for spectra from days 1 and 2 ($R=0.45$, $p<0.0001$) and days 1 and 28 ($R=0.44$, $p<0.0001$) (Figs. 3.8 – 3.9). There were variations among spectra for each solution between days 1 and 2 ($R=0.42$, $p<0.0001$) and between days 1 and 28 ($R=0.31$, $p<0.0001$) (Figs. 3.8 – 3.9). All concentrations were significantly different among spectra for alanine for days 1 and 2 and days 1 and 28, and glycine for days 1 and 2 (Table 3.7a -b). Significant separation of glycogen and glycine, and alanine and glycine, at 60μM and 0.6μM over the experimental period was also shown (Tables 3.8 – 3.9).

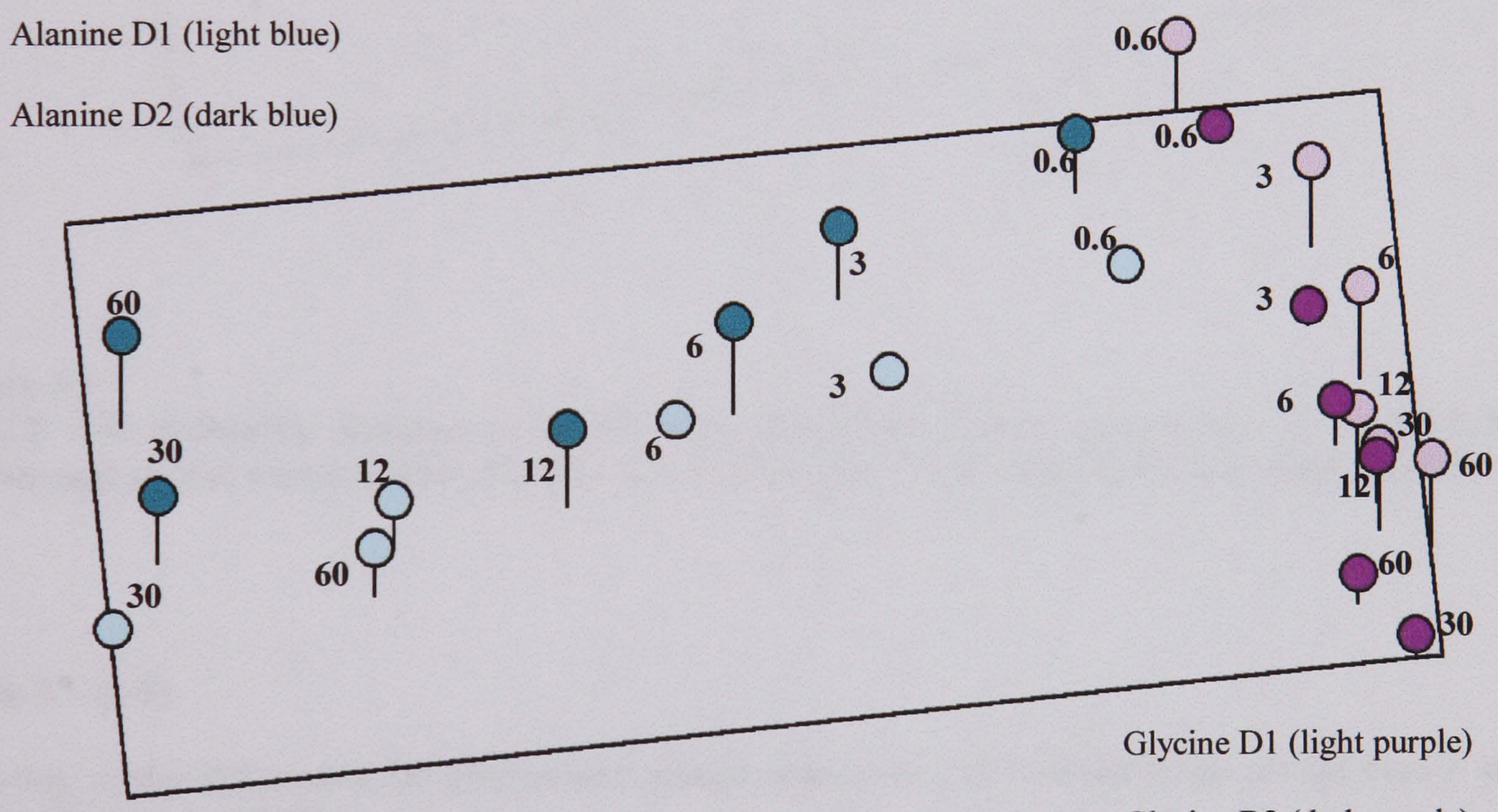


Figure 3.8
PC-CV 3-D ordination diagram of pyrolysis mass spectrometry data (means, n = 3) obtained for alanine and glycine solutions (60 μM, 30 μM, 12 μM, 6 μM, 3 μM and 0.6μM) from days 1 and 2.

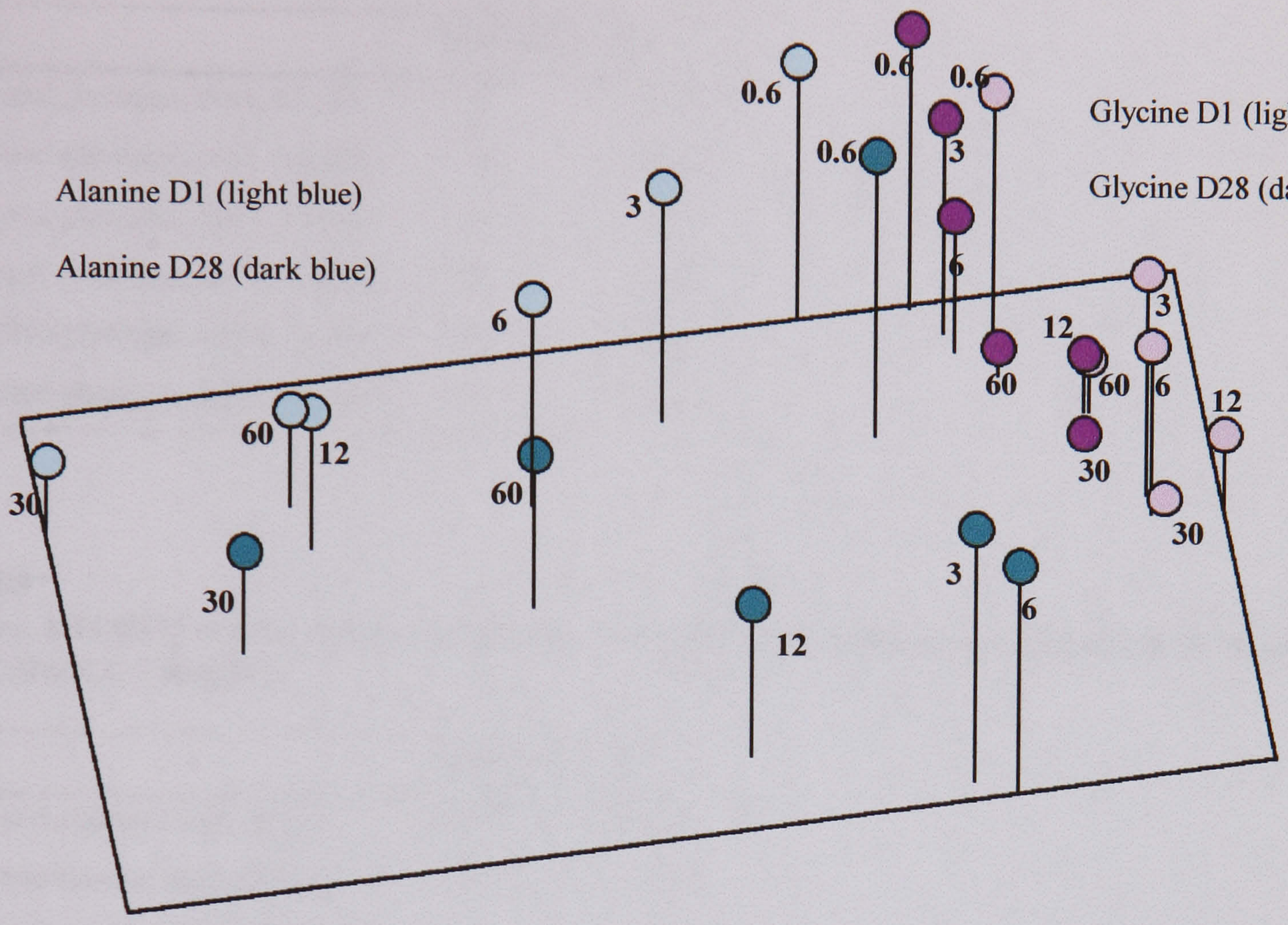


Figure 3.9
PC-CV 3-D ordination diagram pyrolysis mass spectrometry data (means, n = 3) obtained for alanine and glycine solutions (60 μM, 30 μM, 12 μM, 6 μM, 3 μM and 0.6μM) from days 1 and 28.

Table 3.7 (a-b)

One-way ANOSIM results for glycine and alanine comparing all concentrations at: (a) days 1 and 2, and (b) days 1 and 28.

(a) Days 1 and 2

Factor	Global R	p
Glycine Day 1	0.26	< 0.03
Alanine Day 1	0.16	< 0.02
Glycine Day 2	0.16	< 0.02
Alanine Day 2	0.24	< 0.007

(b) Days 1 and 28

Factor	Global R	p
Glycine Day 1	0.18	< 0.01
Alanine Day1	0.16	< 0.02
Glycine Day 28	0.024	> 0.05 (= 0.065)
Alanine Day28	0.30	< 0.003

Table 3.8
One-way ANOSIM results comparing glycine and glycogen solutions at concentrations of 60 μM and 0.6 μM (days 1, 2 and 28).

Factor	Global R	p
Glycine and glycogen, day1, 60 μM	1.00	< 0.05
Glycine and glycogen, day1, 0.6 μM	0.56	< 0.05
Glycine and glycogen, day 2, 60 μM	1.00	< 0.05
Glycine and glycogen, day 2, 0.6 μM	1.00	< 0.05
Glycine and glycogen, day28, 60 μM	1.00	< 0.05
Glycine and glycogen, day28, 0.6 μM	0.93	< 0.05

Table 3.9
One-way ANOSIM results comparing glycine and alanine solutions at concentrations of 60 μM and 0.6 μM (days 1, 2 and 28).

Factor	Global R	p
Glycine and alanine, day1, 60 μM	0.30	< 0.05
Glycine and alanine, day1, 0.6 μM	0.56	< 0.05
Glycine and alanine, day 2, 60 μM	0.41	< 0.05
Glycine and alanine, day 2, 0.6 μM	0.70	< 0.05
Glycine and alanine, day28, 60 μM	0.53	< 0.05
Glycine and alanine, day28, 0.6 μM	0.56	< 0.05

3.4 Discussion

The ability of PyMS to discriminate between chemically similar samples at various concentrations was shown in this study. Good reproducibility between replicate samples of the same concentration among days was also observed. The short-term reproducibility of PyMS data was illustrated by the reproducible separation of the five sample types examined (ANOSIM, R= 0.87, $p<0.0001$). Discrimination between different sample groups was most obvious at concentrations of 60 μM (ANOSIM, R=0.94, $p<0.0001$) of each of the five solutions analysed here (Fig. 3.4). Windig *et al.* (1979) suggested that the spectra for small samples differ

significantly from the spectra for larger quantities of the respective substances, and they attributed this difference to the relatively large influence of background noise in the system at lower concentrations. In this study, PyMS analysis of sample concentrations at 0.6μM resulted in total ion counts that were similar to the background level, but overall discrimination between different samples was still possible at this concentration (ANOSIM, $R=0.088$, $p<0.0001$). Statistically significant separation at all concentrations was also achieved for alanine and glycine, which differed in their chemical composition by virtue of one methyl group only. These results clearly illustrate a high sensitivity of the PyMS technique and suggest that discrimination is possible at lower sample concentrations than previously thought. The results presented here are more in agreement with Freeman *et al.* (1997), who challenged the PyMS technique with increasing levels of biological difference of two closely-related bacterial populations and reported that large biological or chemical differences between samples resulted in large PyMS differences. The PC-CV analyses presented here showed that discrimination between samples was more obvious at higher concentrations than low and where chemical differences between the solutions were greatest. The sensitivity of the PyMS technique was highlighted by the PC-CV discrimination of all groups at the 0.6μM concentration, where sample groups were recovered in distinct clusters corresponding to the five solutions analysed (Fig. 3.5). Although there was evidence of some machine drift among samples as within-group variation between sample days and reduced reproducibility between identical samples, this did not affect the between-group discrimination of different samples. Manchester *et al.* (1995), who showed sustainable separation of five strains of *Carnobacterium* species over a five-week period, also reported that greater reproducibility was observed at shorter time intervals and at higher concentrations.

Comparison of the PyMS spectra obtained for different sample solutions from different days showed that separation into distinct clusters was conserved throughout the experimental period.

Although the spectra obtained for identical sample types were statistically different throughout the experimental period, it is suggested that within-group variability was less significant than between-group variability for all concentrations. This was illustrated by the comparison of alanine and glycine at concentrations of 60 μM and 0.6 μM for days 1 and 2 and days 1 and 28. The effect of instrumental drift is more apparent at the longer time interval and at lower concentrations suggesting that caution should be used when interpreting PyMS data and that concentrations much higher than background levels should be used in order to achieve confidence in the results. Freeman *et al.* (1995) recommended a total ion count in the range 5 x 10^5 and 6 x 10^6. That differences between spectra of identical samples exist is an inherent problem in PyMS (Goodfellow *et al.*, 1997). However, the use of PC-CV analysis to determine between-group variation as compared to within-group variation largely overcomes this problem, particularly when all samples are analysed within a single batch run, and confidence may be placed in the results achieved.

Overall, the results of this study demonstrated that PyMS is a sensitive technique, and resulted in the accurate discrimination of samples at concentrations as low as 0.6 μM (sample size 5 μl). This showed agreement with other studies, which suggested that the PyMS system is robust enough to provide meaningful results within a batch run. The sensitivity of the PyMS technique demonstrated here also shows the potential for examining samples (e.g., groups of organisms) which are closely related in either chemical structure or taxonomy. Furthermore, since the solutions investigated in this study were simple organic compounds, it could be concluded that the between-run variation observed was attributable to instrumental drift and not growth. This type of variability could therefore be minimised in future work by analysing all samples within a single batch run and at least three replicates of each sample. It may also be safe to assume that more complex organic material, in the form of whole cell organisms, may provide spectra that

are complex enough to allow discrimination and that concentrations of such material would produce total ion counts at levels well above background. In the analysis of whole cells it may be expected that differences between samples would be greater than those encountered in this experiment.

Chapter 4

Evaluation of the Discriminatory Capacity of PyMS with respect to Variation in Environmental Condition I: Fucoid algae

4.1 Introduction

Marine macroalgae, particularly representatives of the order Fucales (Phaeophyceae), dominate the intertidal across rocky shores of the British Isles and exhibit natural variation in phenotype in response to differences in habitat. Habitat-induced variation has been particularly well studied in the Phaeophyceae and has caused problems in taxonomic discrimination (Powell, 1963). Classification of the Phaeophyceae (Kylin, 1917) is still based largely on diagnostic characters including morphology, life history and physiology (Bold & Wynne. 1978; Clayton, 1984).

The construction of a molecular phylogeny of the Fucales has only recently been attempted (Rousseau, *et al.*, 1997; Rousseau & de Reviers, 1999) and evolutionary relationships within the order remain controversial (Serrão *et al.*, 1999). Taxa within this group have been the subject of many investigations with regard to their taxonomic and ecological relationships (e.g., Burrows & Lodge, 1954; Russell, 1978; Rice & Chapman, 1985; Wynne & Magne, 1991; Tan & Druehl, 1993). Many species within the order Fucales exhibit intraspecific phenotypic and physiological plasticity in response to variations in environmental condition (Russell, 1978, 1979; Norton *et al.*, 1981). This natural variability is particularly apparent in the genus *Fucus* (Niemeck & Mathieson, 1976; Schonbeck & Norton, 1981). Experimental techniques used to investigate the significance and basis of this variability have included detailed seasonal observations of *in situ* populations (Kalvas & Kautsky, 1993), reciprocal transplantation and culturing experiments (Sideman & Mathieson, 1985; McLachlan *et al.*, 1971), and genetic and hybridisation studies (Mathieson *et al.*, 1981; Scott & Hardy, 1994).

Pyrolysis techniques have previously been applied to algal taxonomic investigations with some success. Nichols *et al.* (1968) used pyrolysis-gas-liquid chromatography (Py-GC) to investigate

specific problems of identification and characterisation among taxa of the Chlorophycean and Rhodophycean algae. Major differences were found to exist between genera while fewer differences existed between species of the same genus or different geographical isolates of the same species (Nichols *et al.*, 1968). Py-GC was also applied by Bird *et al.* (1987) as a taxonomic tool in investigations of species of *Gracilaria* (Rhodophyceae) which are known to differ in the chemical structure of their extracted agar. The composition of the extracted agar was used to discriminate taxa that were not easily differentiated by morphological or other conventional criteria (Bird *et al.*, 1987). Russell (1995) applied pyrolysis mass spectrometry (PyMS) to the study of brown algal systematics; he successfully discriminated between *Pilayella littoralis* sampled from different geographical locations, and concluded that this confirmed their position as separate subspecies. Russell (1995) also noted that the technique failed to clearly separate *Fucus serratus* and *Fucus vesiculosus* collected from the study site concerned and he considered this result to be confirmation of the existence of putative hybrids between these two co-existing species. PyMS has also been used to support morphological evidence of hybridisation between *Fucus spiralis* and *Fucus vesiculosus* (Hardy *et al.*, 1998). Scott *et al.* (in press) recently used this technique in parallel with morphological investigations to resolve the ecological position *of F. spiralis forma nanus*, the dwarf form of *F. spiralis*. Despite these broad-ranging investigations using PyMS as an analytical tool, it appears that the potential for the routine use of PyMS as an ecological tool has not been fully evaluated.

Standardisation of growth conditions of samples prior to pyrolysis has been proposed as one means of minimising the effect of biological variation between identical samples (Voorhees et al., 1988). However this is not always possible with material sampled directly from the environment. The purpose of this study was to investigate how robust the PyMS technique is for the analysis of samples taken directly from the environment. In order to determine how sensitive

PyMS was to any differences in sample composition, due to variation in habitat, the systematic relationships of fucoid algae collected from a number of different sites were investigated. Fucoid algae provide a good model for this type of investigation owing to their abundance on British shores and well-documented phenotypic variability in response to habitat condition. The widely-reported occurrence of putative hybrids between various taxa and unresolved evolutionary relationships render this group an interesting subject for study.

British representatives of taxa within the order Fucales were investigated in this study. These included eight genera belonging to four of the six families currently recognised as belonging to the order (Rousseau & de Reviers, 1997). The objectives of this study were to use PyMS to compare species collected from rocky shores of the British Isles, which are characterised by fluctuating environmental conditions, at three levels:

1. Replicates of similar species within a shore.
2. Similar species collected from two different shores from within one site.
3. Similar species from sampled from three different shores located at two geographically distinct sites.

Samples were freeze-dried for storage prior to analysis, therefore the effect of sample preparation prior to analysis, namely the effect of freeze-drying samples, was also investigated. Analysis by PyMS of different parts of the same organism was also investigated.

4.2 Materials and Methods

4.2.1 Sample collection

Samples of mature plants from the brown algal class Phaeophyceae were collected from 5 shores located at four geographically distinct sites of the British Isles (Fig. 4.1, Table 4.2). These were St. Mary's Island, Tyne and Wear (Grid reference NZ347753 and NT350749) (hereafter shores 1 and 2 respectively), Berwick upon Tweed, Northumberland (Grid reference NT9953) (hereafter shore 3), Wembury Bay, Devon (Grid reference SX5147) (hereafter shore 4) and Atlantic Bridge, Clachan, Argyll (Grid reference NM7819) (hereafter shore 5).

Shores 1, 2 and 3 were north-east facing, moderately exposed and consisted mainly of rocky platform with sand, small stones and boulders, and were covered with dense growth of fucoid algae at all levels. Shore 4 was a southerly-facing, moderately exposed shore, characterised by an abundance of rockpools, dense growth of rhodophycean algae and sporadic cover of fucoid algae. Shore 5 was estuarine, very steeply sloping and was not characterised by the typical zonation pattern of *Fucus* species seen at the other shores in this study. *Fucus spiralis*, *Pelvetia canaliculata* and *Ascophyllum nodosum* were found growing in dense patches at shore 5.

All collections were made by randomly selecting a point at the centre of each species zone on the shore and sampling the 5 nearest plants. Representatives of each species were identified using the characters described by Schueller & Peters, 1994 (Table 4.1). Care was taken to select typical type-specimens only using the characteristics outlined in Table 4.1. Five representatives of each of the following species were sampled over two consecutive tides from shores 1, 2 and 3: *Ascophyllum nodosum* (Fucales, Fucaceae), *Fucus vesiculosus* (Fucales, Fucaceae), *Fucus serratus* (Fucales, Fucaceae), *Fucus spiralis* (Fucales, Fucaceae), *Laminaria digitata*

(Laminariales, Phaeophyceae) and *Chondrus crispus* (Rhodophyceae). Additionally, 5 individuals of the species *Himanthalia elongata* (Fucales, Himanthaliaceae) and *Halidrys siliquosa* (Fucales, Cystoseiraceae) were collected from shore 3; these species were not present on shores 1 and 2. Samples were transported to the laboratory in clean polythene bags and frozen on the day of collection. Three representatives of each of the species *Bifurcaria bifurcata* (Fucales, Cystoseiraceae), *Cystoseira baccata* (Fucales, Cystoseiraceae) and *Sargassum muticum* (Fucales, Sargassaceae), all of which have a southerly distribution in the British Isles (Norton, 1985), were sampled from shore 4. Five individuals of *Ascophyllum nodosum mackaii* (Fucales, Fucaceae), which is rarely found on British shores, were collected from shore 5 only. Samples were stored at -20° C until they were processed.

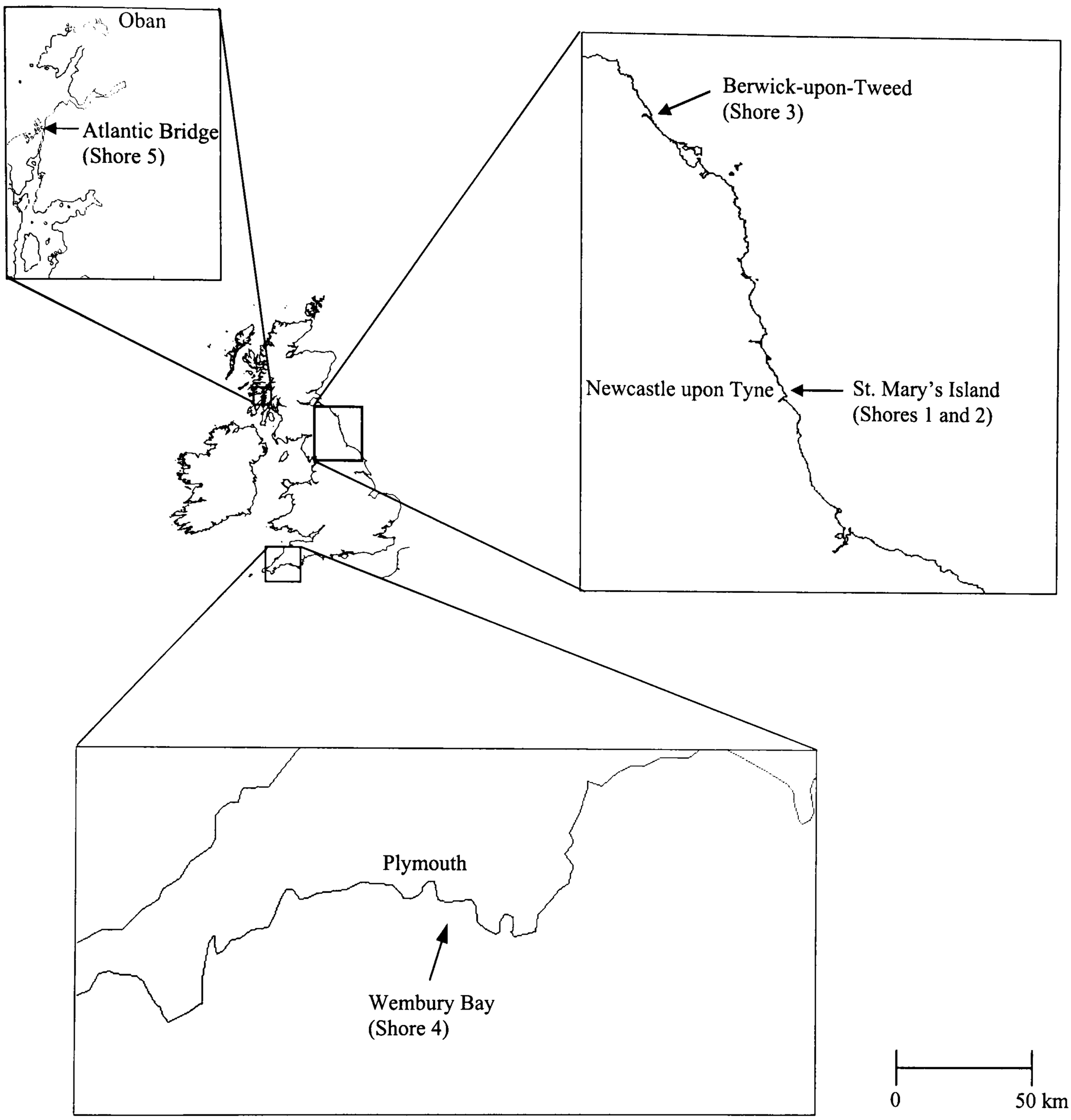


Figure 4.1
Locations of the five shores around the British Isles from which samples were collected.

Table 4.1
Main morphological characteristics of *Fucus* species investigated (after Schueller & Peters, 1994)

Character	*F. ceranoides*	*F. serratus*	*F. spiralis*	*F. vesiculosus*
Margins	entire	serrate	entire	entire
Midrib	distinct	distinct	distinct	distinct, pronounced
Air bladder	absent	absent	absent	present
Texture	delicate	firm	firm	firm
Receptacle morphology	short, pointed	long, flat	short, swollen, with sterile rim	short, flat or swollen
Reproduction	dioecious	dioecious	monoecious	dioecious

4.2.2 Sample preparation

Thallus sections of each of the samples were washed with distilled water to remove any large debris before the meristoderm was removed (in order to mitigate any contamination from epiphytes) with a clean scalpel blade. The thalli were finely ground in a pestle and mortar, under liquid nitrogen, before being freeze-dried. Between each sample preparation the pestle and mortar and scalpel blade were washed with Decon-90, rinsed with 10 % hydrochloric acid and then with distilled water, and flamed with ethanol. Freeze dried samples were stored in clean glass vials, in a desiccator, until they were analysed. The effect of freeze-drying on sample discrimination was investigated by comparing two sets of identical samples; one sample in each pair had been freeze-dried and the other freshly frozen.

4.2.3 Pyrolysis mass spectrometry

All analyses were carried out using the RaPyD-400 system as described in section 2.1.1. Samples were re-hydrated with distilled waster prior to analysis and a small amount of each was smeared onto clean 530° C curie-point foils, held in glass pyrolysis tubes, using a disposable plastic loop. Six replicates from each sample were prepared. Analysis of the samples was then carried out under the control of a computer as previously described (section 2.1.2 – 2.1.4).

Table 4.2
Algal species included in the study (in alphabetical order within families) with geographical location, shore and collection date.

Family	Species and taxonomic authorities	Location and shore	Date collected
Cystoseiraceae	*Bifurcaria bifurcata* Ross	Wembury Bay, Devon, shore 4	11/10/98
Cystoseiraceae	*Cystoseira baccata* (Gmelin) Silva	Wembury Bay, Devon, shore 4	11/10/98
Cystoseiraceae	*Halidrys siliquosa* (Linnaeus) Lyngbye	Berwick, Northumberland, shore3	13/8/98
Fucaceae	*Ascophyllum nodosum* (Linnaeus) Le Jolis	St. Mary's Island, Tyne and Wear, shores 1 and 2	14/8/98
		Berwick, Northumberland, shore 3	13/8/98
Fucaceae	*Ascophyllum nodosum mackaii* (Linnaeus) Le Jolis	Atlantic Bridge, Clachan, Argyll, shore 5	21/4/98
Fucaceae	*Fucus serratus* Linnaeus	St. Mary's Island, Tyne and Wear, shores 1 and 2	14/8/98
		Berwick, Northumberland, shore 3	13/8/98
Fucaceae	*Fucus spiralis* Linnaeus	St. Mary's Island, Tyne and Wear, shores 1 and 2	14/8/98
		Berwick, Northumberland, shore 3	13/8/98
Fucaceae	*Fucus vesiculosus* Linnaeus	St. Mary's Island, Tyne and Wear, shores 1 and 2	14/8/8
		Berwick, Northumberland, shore 3	13/8/98
Fucaceae	*Pelvetia canaliculata* (Linnaeus) Decaisne et Thuret	St. Mary's Island, Tyne and Wear, shores 1 and 2	14/8/98
Fucaceae	*Fucus ceranoides* Linnaeus	Atlantic Bridge, Clachan, Argyll, shore 5	21/04/98
Himanthaliaceae	*Himanthalia elongata* (Linnaeus) S.F.Gray	Berwick, Northumberland, shore 3	13/8/98
Sargassaceae	*Sargassum muticum* (Yendo) Fensholt	Wembury Bay, Devon, shore 4	11/10/98

4.2.4 Species discrimination

Species were compared by PyMS at three levels of discrimination:

1. Within each of shores 1 and 2, replicates of the species *F. serratus*, *F. vesiculosus*, *F. spiralis*, *A. nodosum* and *P. canaliculata*, were analysed to determine any variability detectable by PyMS between replicates sampled from the same shore.

2. Between shores 1 and 2, replicates of the species *F. serratus*, *F. vesiculosus*, *F. spiralis*, *A. nodosum* and *P. canaliculata*, were analysed to determine any intraspecific variability detectable by PyMS for species sampled from two shores within one geographical site (less than 50 km apart).

3. Between shores 1, 2 and 3, replicates of the species *F. serratus*, *F. vesiculosus*, *F. spiralis* and *A. nodosum*, were analysed to determine any intraspecific variability detectable by PyMS for species sampled from shores which were geographically separated by more than 50 km .

Species were selected for the above analyses based on their occurrence at each shore. *P. canaliculata* was not found at shore 3 and was therefore omitted from analysis (3) above.

4.2.5 Taxonomic applications

The vegetative and reproductive forms of *Himanthalia elongata* were included in one analysis to investigate the PyMS discrimination between different parts of an organism. Analysis of all species collected within the order Fucales was also performed; this analysis included two putative morphs of the species *Ascophyllum nodosum* (*A. nodosum* and *A. nodosum mackaii*). *Laminaria digitata* and *Chondrus crispus* samples were included in each batch run to test the discriminatory capacity of PyMS.

4.2.6 Data analysis

Pre-processing of sample data was carried out as described in section 2.2.1. Principal components analysis was then performed followed by canonical variates analysis. Similarity dendrograms showing hierarchical group clustering resulting from PC-CV analysis were then drawn using the UPGMA average linkage clustering algorithm. Two-way crossed and one-way ANOSIM were performed on species comparisons, within and between shores, and between replicates of species using the PRIMER v4.0 software package (Plymouth Marine Laboratory, NERC, 1994).

4.3 Results

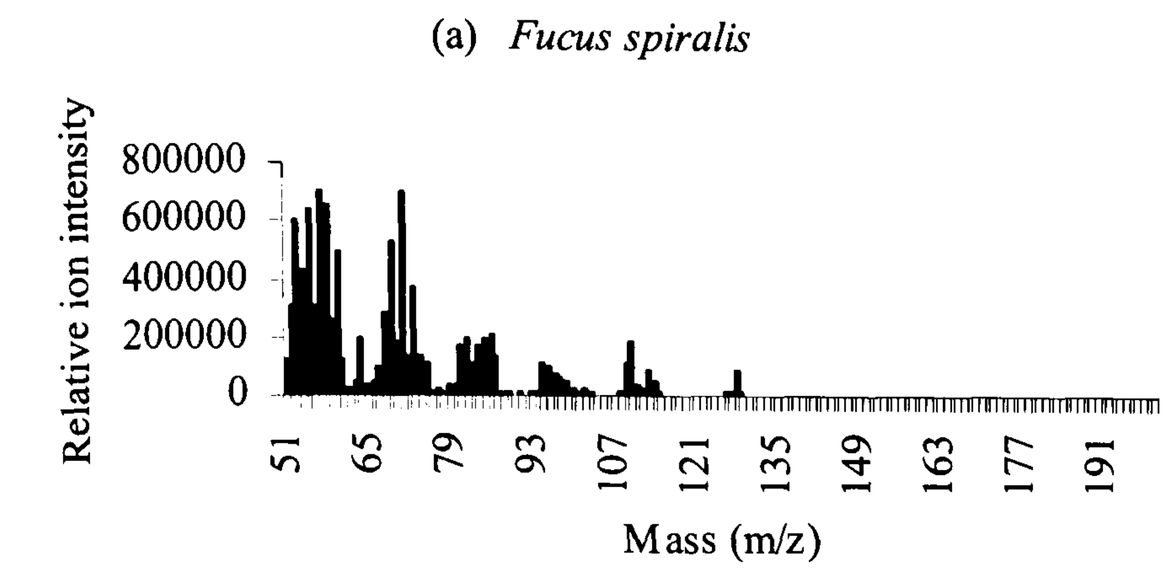
Pyrolysis mass spectra were recorded for all species analysed at integrated ion counts in the mass:charge range 51-200 (Fig. 4.2 a-l).

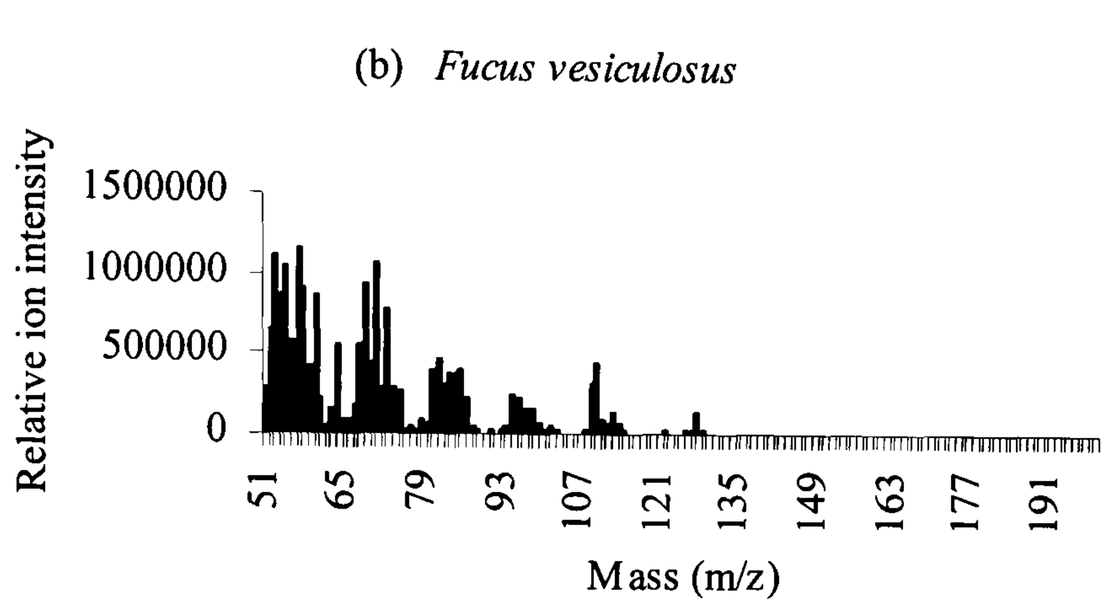
4.3.1 Sample preparation

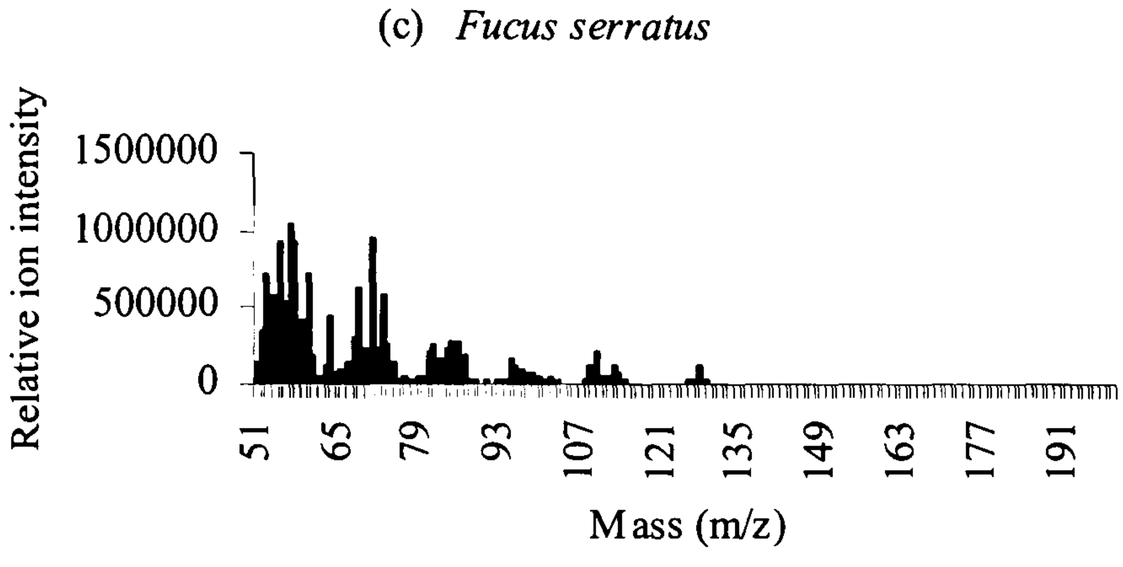
No significant effect of freeze-drying samples on discrimination among species was detectable by PyMS (ANOSIM, R=-0.051, p=0.98).

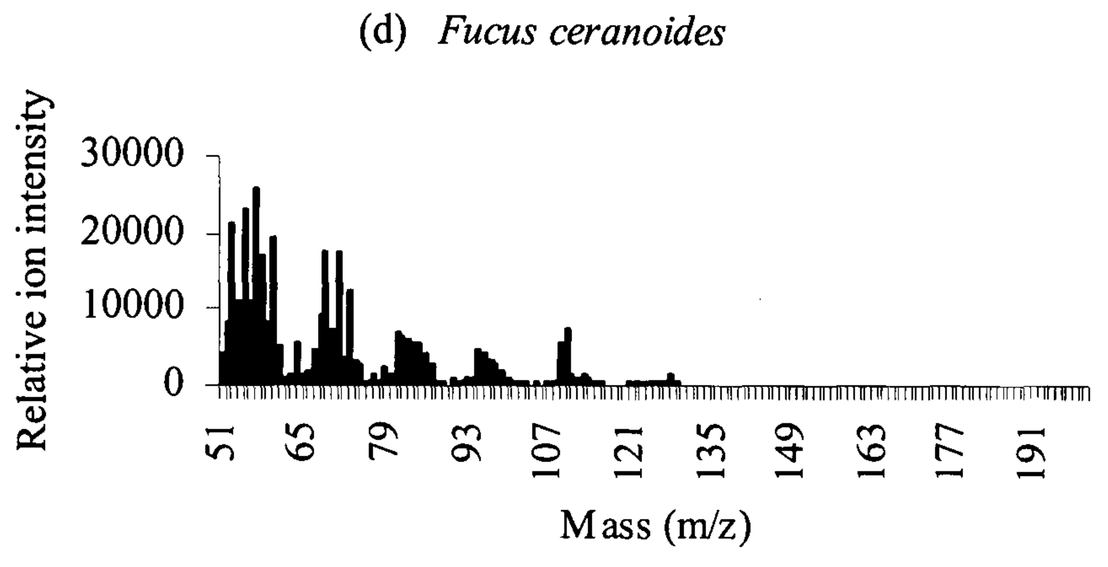
4.3.2 Discrimination between species

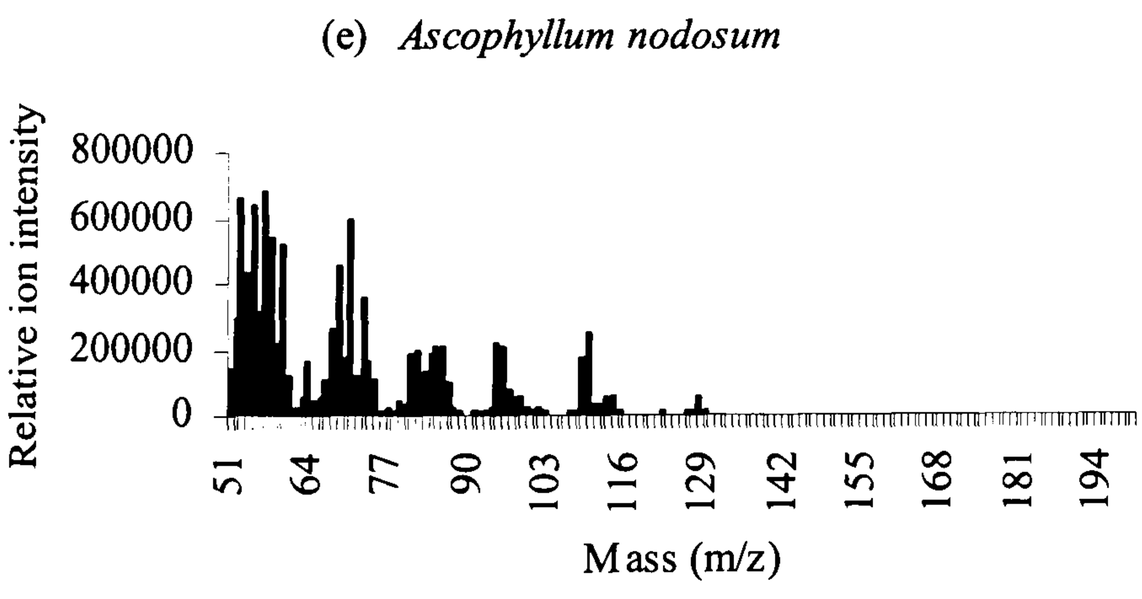
In all analyses, *L. digitata* and *C. crispus* were clearly distinguished from all other samples (two-way ANOSIM, R=0.857, p<0.0001). ANOSIM pairwise tests showed a Global R value of >0.99 (p<0.0001) for *L. digitata* and *C. crispus* in comparisons with all other species (Table 4.2). For clarity, *L. digitata* and *C. crispus* were removed from the subsequent analyses. There was no significant difference detectable by PyMS between replicates of species analysed from within shore 1 (ANOSIM R=0.003, p=0.42) and all species were shown to be significantly different from each other (ANOSIM R=0.71, p<0.0001) (Fig 4.3). Similarly, replicates of species were not significantly different among individuals analysed from within shore 2 (ANOSIM, R=0.0001, p=0.49) and shore 3 (ANOSIM, R=0.046, p=0.23). Species collected from within shore 2 and within shore 3 were also shown to be distinct from each other (ANOSIM, R=0.36, p<0.0001 and ANOSIM, R=0.712, p<0.0001 respectively) (Figs. 4.4 – 4.5).











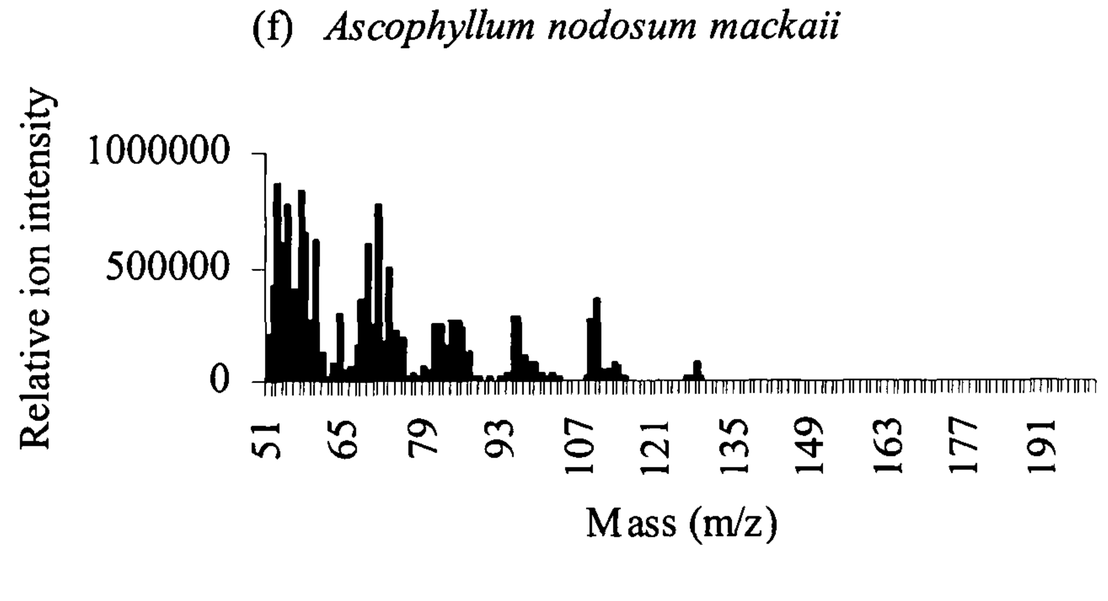
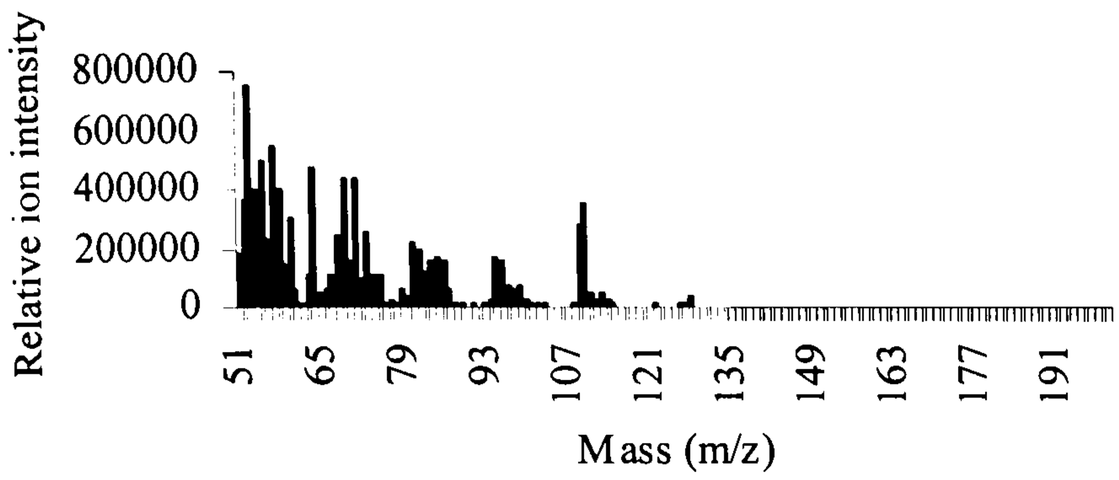
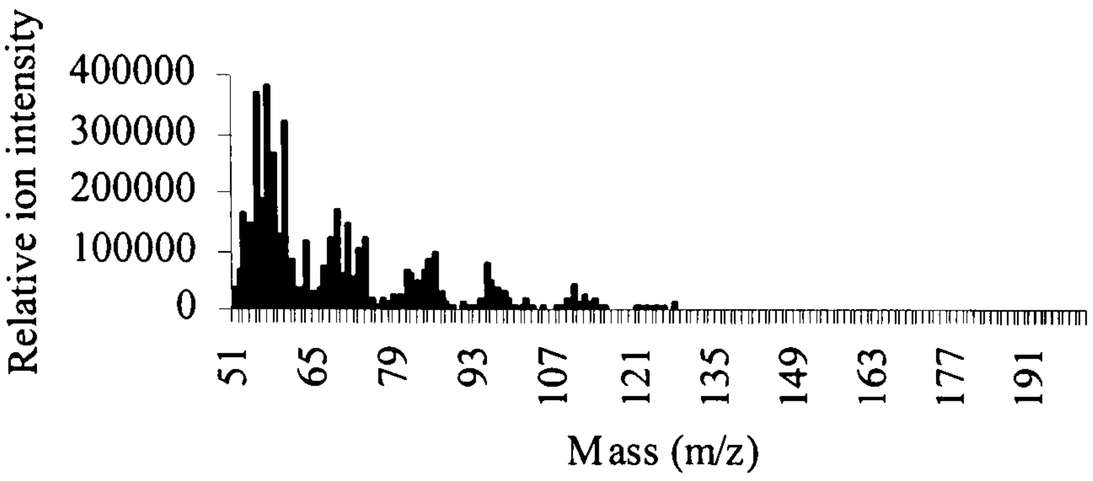


Figure 4.2 (a-f)
Examples of pyrolysis mass spectra collected for sample taxa representative of the order Fucales.

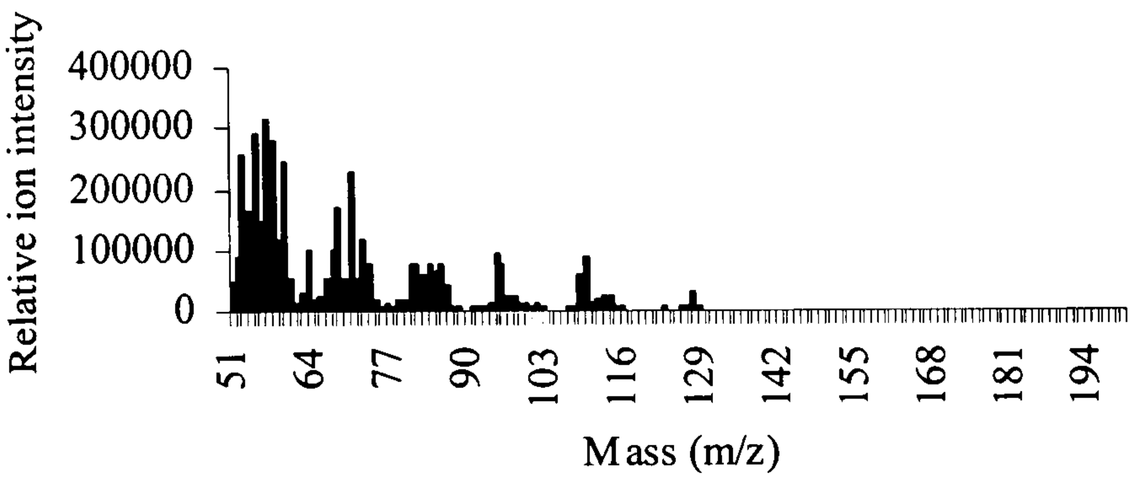
(g) *Pelvetia canaliculata*



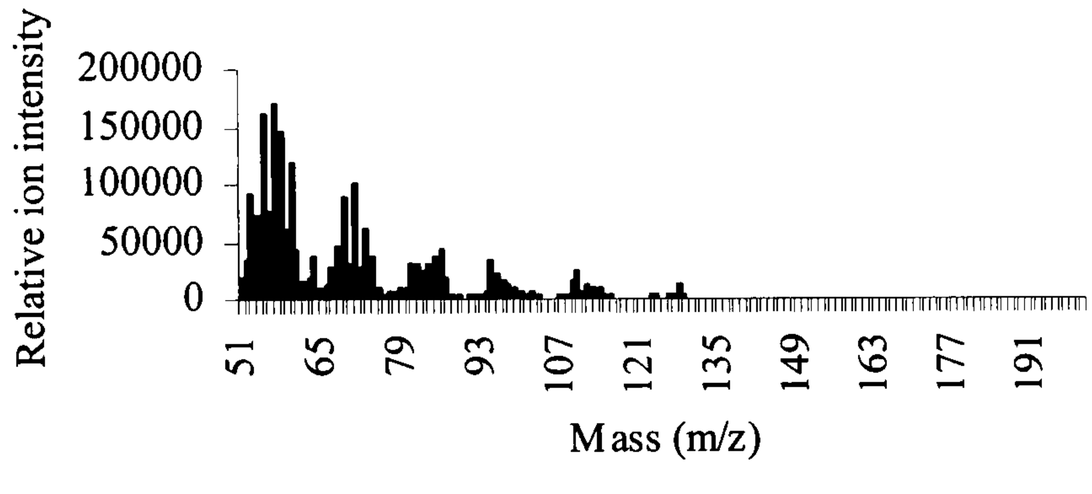
(h) *Himanthalia elongata*



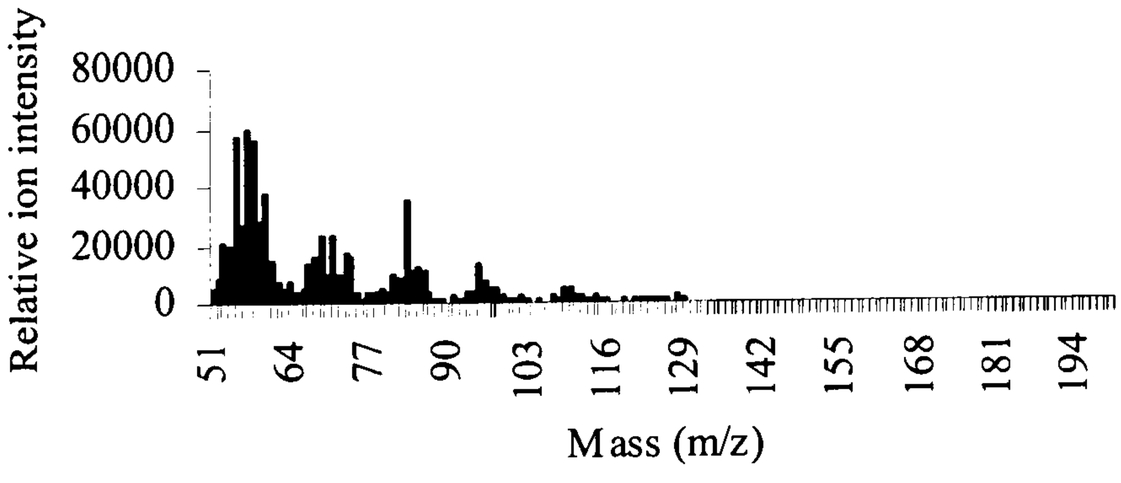
(i) *Halidrys siliquosa*



(j) *Sargassum muticum*



(k) *Cystoseira baccata*



(l) *Bifurcaria bifurcata*

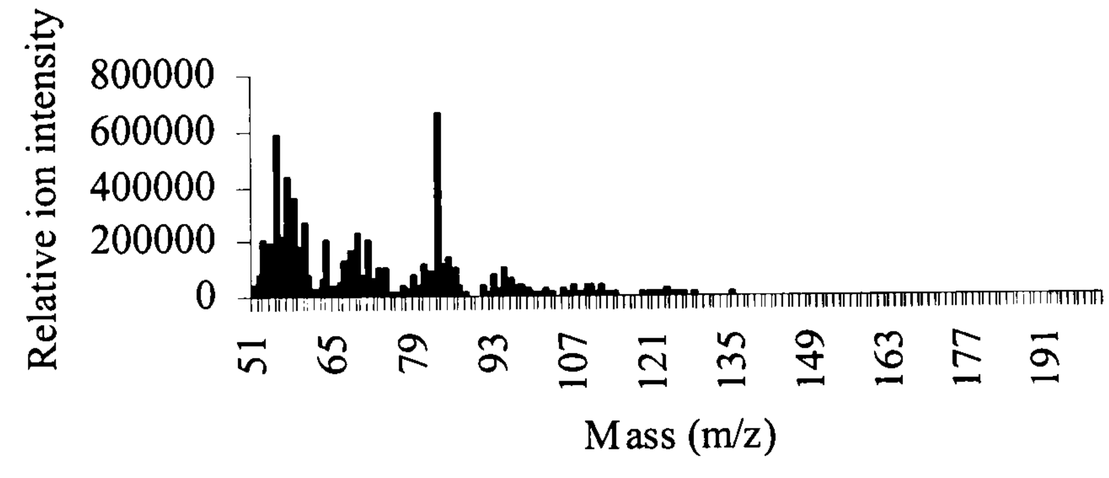


Figure 4.2 (g-l)
Examples of pyrolysis mass spectra collected for sample taxa representative of the order Fucales.

Table 4.2
Pairwise results from two-way ANOSIM of shore 1 species.

Factor	Global R	P
F. spiralis, F. vesiculosus	0.317	< 0.002
F. spiralis, F. serratus	0.674	< 0.0001
F. spiralis, A. nodosum	0.770	< 0.0001
F. spiralis, P. canaliculata	0.616	< 0.0001
F. spiralis, L. digitata	1.00	< 0.0001
F. spiralis, C. crispus	0.993	< 0.0001
F. vesiculosus, F. serratus	0.734	< 0.0001
F. vesiculosus , A. nodosum	0.968	< 0.0001
F. vesiculosus, P. canaliculata	0.926	< 0.0001
F. vesiculosus, L. digitata	1.00	< 0.0001
F. vesiculosus, C. crispus	1.00	< 0.0001
F. serratus, A. nodosum	0.911	< 0.0001
F. serratus, P. canaliculata	1.00	< 0.0001
F. serratus, L. digitata	1.00	< 0.0001
F. serratus, C. crispus	1.00	< 0.0001
A. nodosum, P. canaliculata	1.00	< 0.0001
A. nodosum, L. digitata	0.970	< 0.0001
A. nodosm, C. crispus	1.00	< 0.0001
P. canaliculata, L. digitata	1.00	< 0.0001
P. canaliculata, C. crispus	1.00	< 0.0001
L. digitata, C. crispus	1.00	< 0.0001

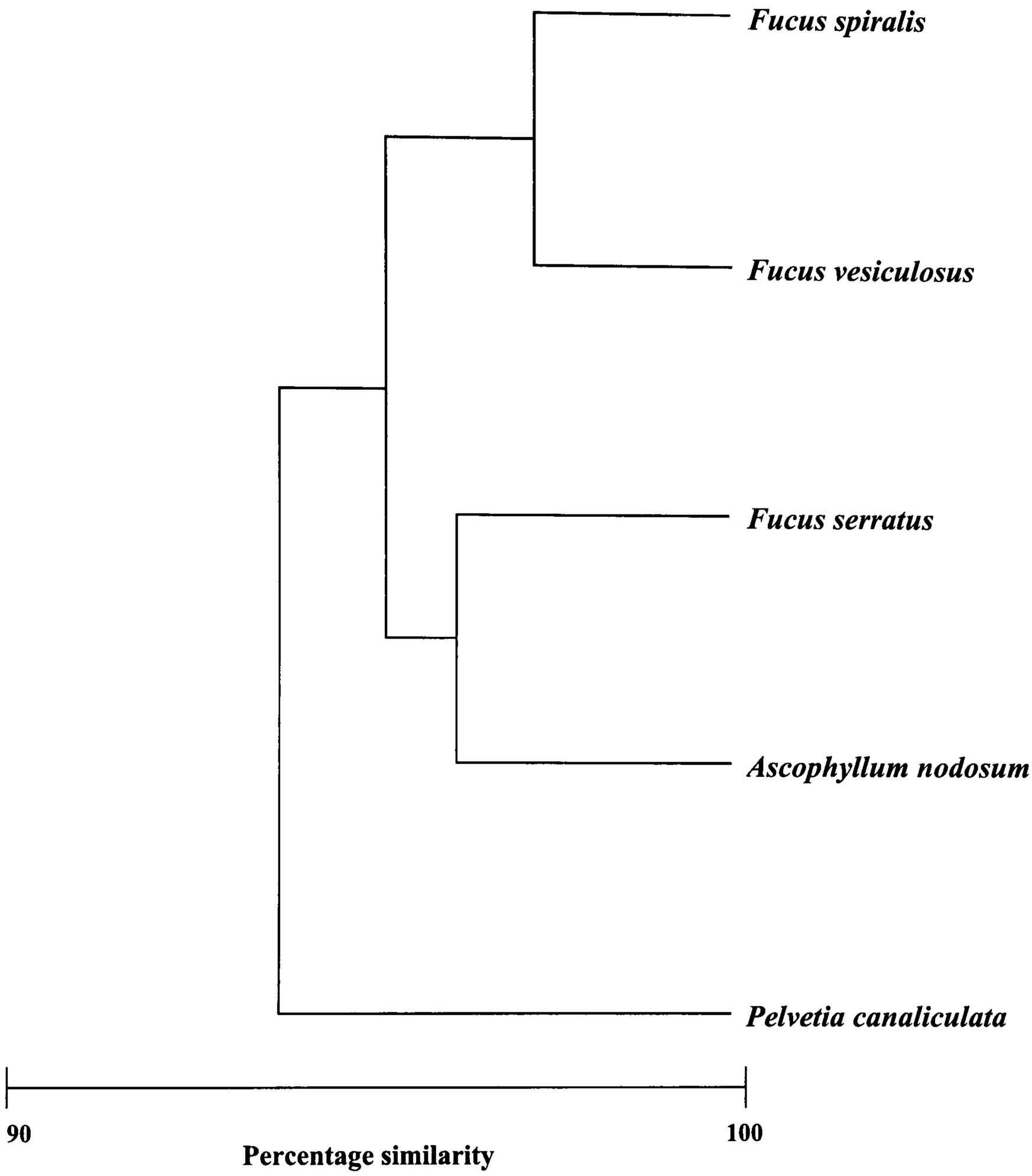


Figure 4.3
PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 5 samples of species collected from shore 1 (St. Mary's Island).

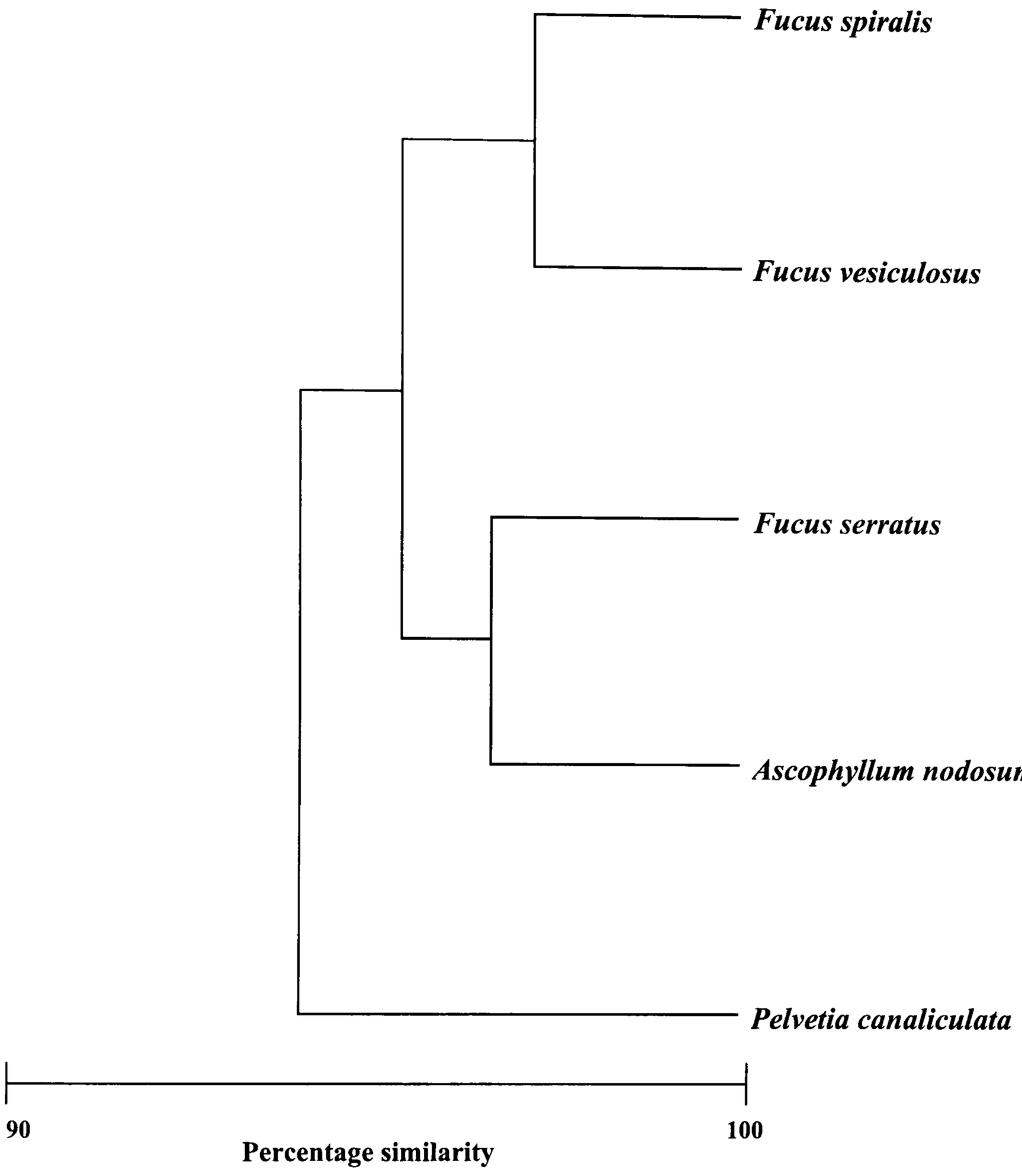


Figure 4.4
PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 5 samples of species collected from shore 2 (St. Mary's Island).

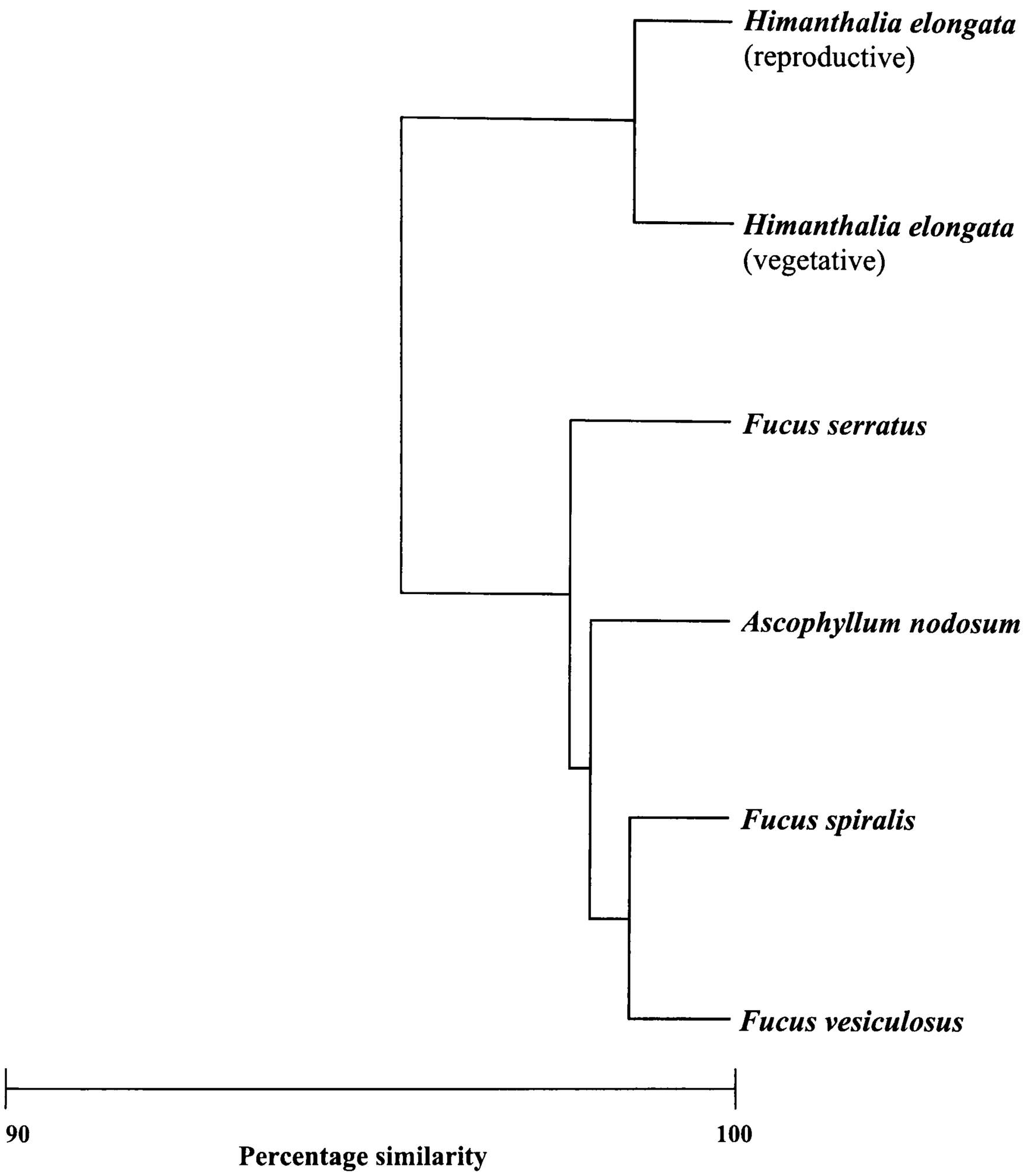


Figure 4.5
PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 5 samples of species collected from shore 3 (Berwick), including vegetative and reproductive forms of *Himanthalia elongata*.

Analyses of variability among similar species collected from two separate shores, within one site (shores 1 and 2), showed no significant between-shore differences (ANOSIM, R=0.115, p=0.36) (Fig. 4.7). One-way ANOSIM of samples from shores 1 and 2 revealed a significant between-shore difference among *Ascophyllum nodosum* individuals (R=0.437, p<0.001) which can be seen in Figure 4.7. Overall between-site intraspecific variability among species, from shores 1, 2 and 3 collectively, was not significant (ANOSIM, R=-0.1, p=0.74) (Fig. 4.8). One-way ANOSIM of samples from shores 1, 2 and 3, clarified that replicates of *Fucus spiralis* were not significantly different between shores 1 and 2 (R=-0.052, p=0.26). However samples of this species between shores 1 and 3 (R=0.337, p<0.0003), and between shores 2 and 3 (R=0.358, p<0.0003), were significantly different. *Fucus vesiculosus* samples similarly showed differences between shores 1 and 3 (one-way ANOSIM, R=0.17, P<0.03) and shores 2 and 3 (one-way ANOSIM, R=0.073, P<0.04). *Fucus vesiculosus* replicates were not significantly different between shores 1 and 2 (One-way ANOSIM, R=0.11, P=0.1). These relationships are shown in Figure 4.8.

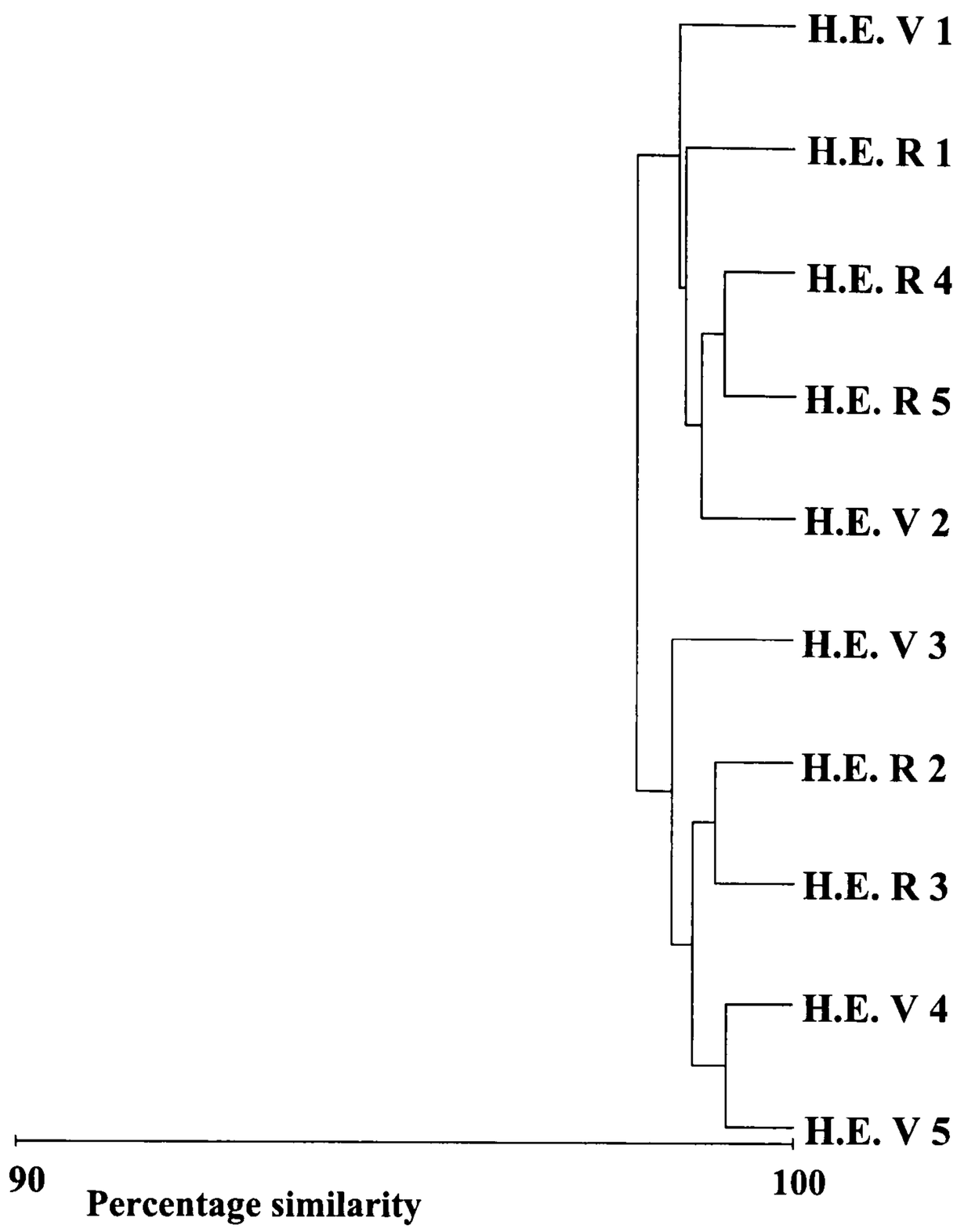


Figure 4.6
PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 5 samples of vegetative (labelled H.E. V 1-5) and reproductive (labelled H.E. R 1-5) forms of *Himanthalia elongata* species collected from shore 3 (Berwick).

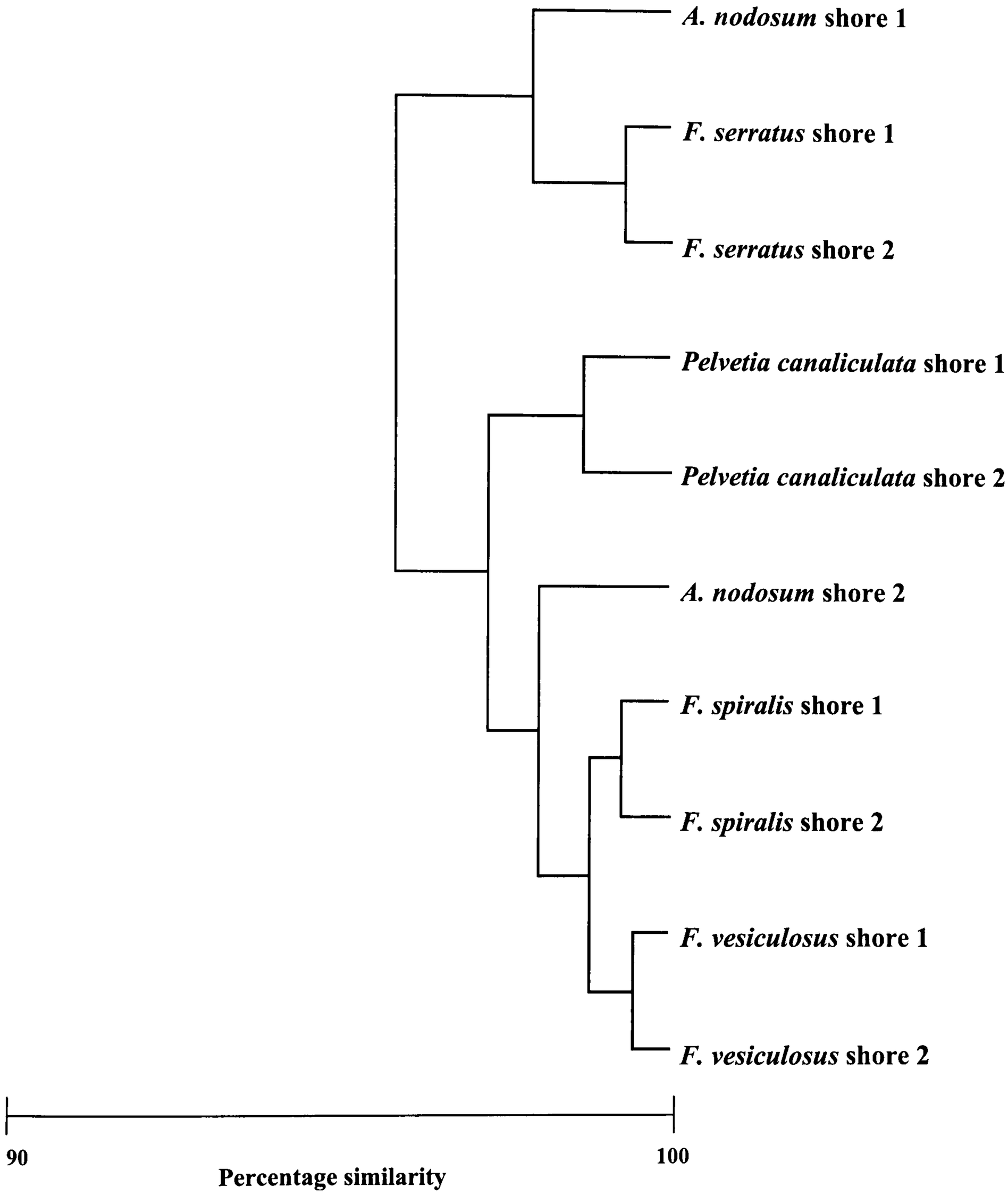


Figure 4.7
PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 5 samples of species collected from shores 1 and 2 (St. Mary's Island).

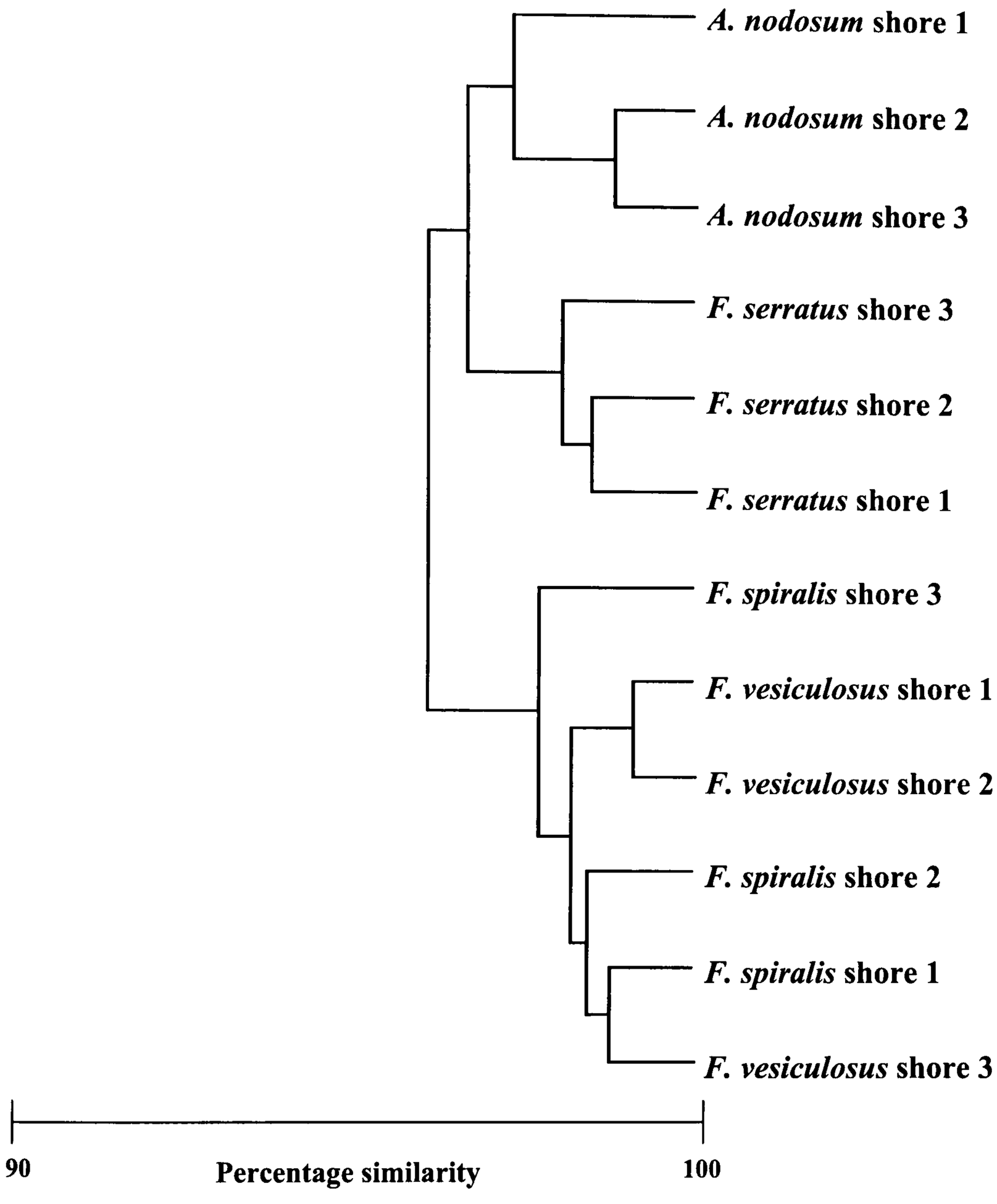


Figure 4.8

PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 5 samples of species collected from shores 1, 2 (St. Mary's Island) and 3 (Berwick).

4.3.3 Taxonomic applications

The PyMS-derived dendrograms presented for species collected from shores 1, 2 and 3 show the similarity between five British common shore species from the Fucaceae, as determined by PyMS (Figs. 4.3 – 4.5).

Samples of the vegetative and reproductive parts of the species *Himanthalia elongata* were distinguished as separate entities by PyMS (one-way ANOSIM, $R=0.154$, $p<0.0065$). The similarity dendrogram for these two forms showed that 2 samples of the vegetative form clustered with the reproductive form cluster and were not from the same plant (Fig. 4.6).

Ascophyllum nodosum and the ecotype *Ascophyllum nodosum mackaii* were also discriminated by PyMS (one-way ANOSIM, $R=0.274$, $p<0.006$) and formed two distinct clusters (Figs. 4.9). These two groups also formed part of the larger Fucaceae group of samples in the similarity dendrogram derived from analysis of all collected species representative of the order Fucales (Fig. 4.10). The investigated Fucales showed one robust clade corresponding to the Fucaceae with the exception of *Himanthalia elongata* which appeared to cluster more closely with another clade which corresponded approximately to a Cystoseiraceae-Sargassaceae group (Fig. 4.10). *Bifurcaria bifurcata*, another member of the Cystoseiraceae, appeared to form a separate branch entirely, while *Halidrys siliquosa*, also a member of the Cystoseiraceae, appeared to be monophyletic with the Fucaceae group, forming a separate branch (Fig. 4.10). Within the Fucaceae cluster, the three common shore *Fucus* species (*F. serratus, F. vesiculosus and F. spiralis*) appeared as a distinct group, closely similar to the *Ascophyllum* group. *Fucus ceranoides*, an estuarine species, was separated from the other *Fucus* species forming a separate branch, which was shown to be more similar to *Pelvetia canaliculata*, a high shore species.

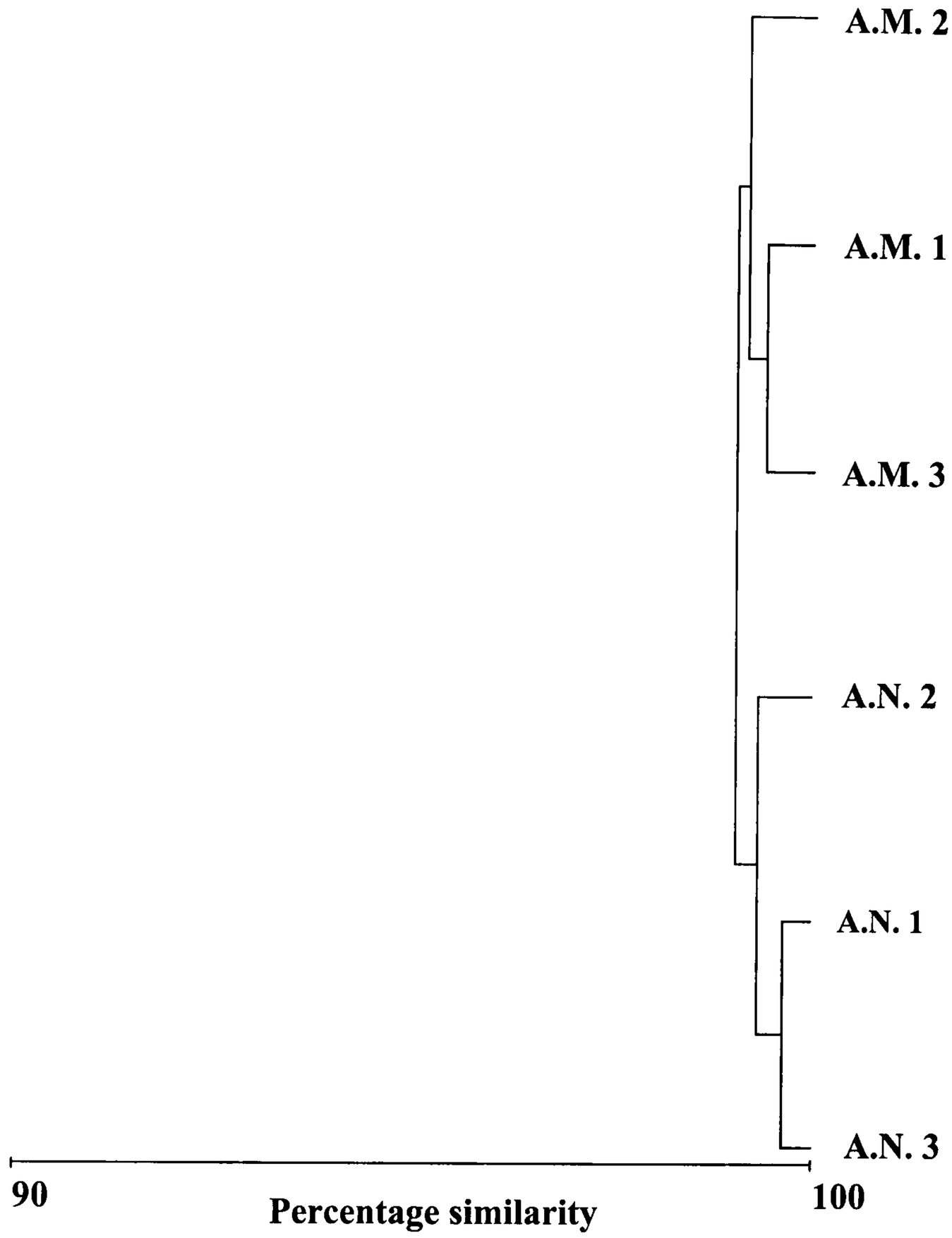


Figure 4.9
PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 3 samples of *Ascophyllum nodosum* (labelled A.N. 1-3) and *Ascophyllum nodosum mackaii* (labelled A.M. 1-3) collected from shore 5 (Atlantic Bridge).

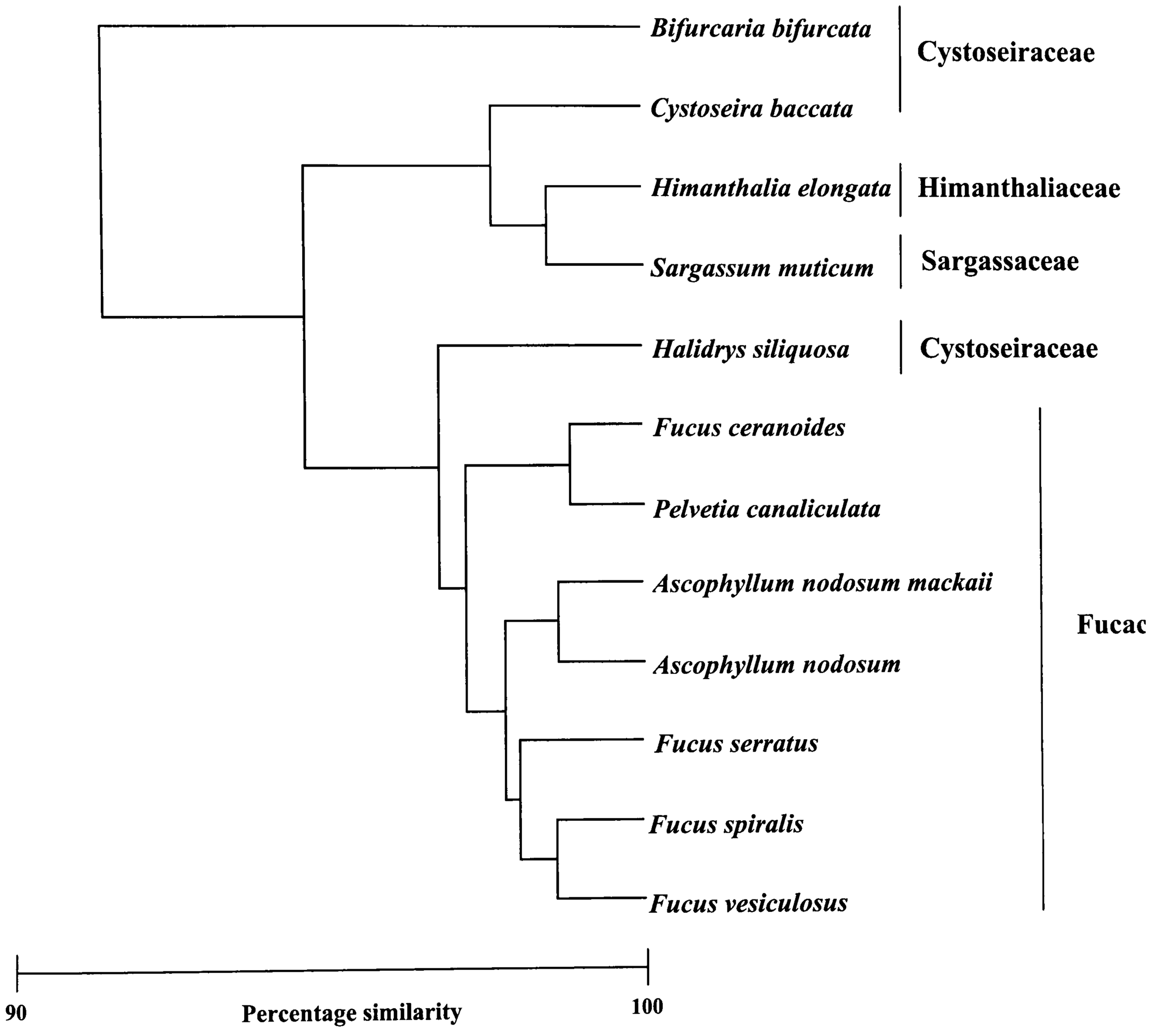


Figure 4.10
PyMS-derived similarity dendrogram from analysis of 6 replicates from each of 3 samples of species, within the order fucales, collected from shores 1, 2 (St. Mary's Island), 3 (Berwick), 4 (Wembury) and 5 (Atlantic Bridge).

4.4 Discussion

This study demonstrated that PyMS could be employed for whole-organism analysis and that these organisms could be rapidly characterised both quantitatively and qualitatively using this technique. Detection of volatiles of the sample algae produced a characteristic 'fingerprint' of each that exhibited sufficient differences during analysis to allow significant separation of the species and genera under investigation. Discrimination among identical samples was not affected by the method of preparation (i.e. freeze-drying) prior to analysis, implying that the characteristics of the sample, which were used for discrimination in PyMS, were conserved during the freeze-drying process. This is relevant to potential use of PyMS for the examination of herbarium samples, or samples from geographically distant locations, in future work. The discriminatory capacity of PyMS was illustrated by the consistent isolation of *L. digitata* and *C. crispus* samples as outlying groups compared to all other taxa, such that these two groups could be eliminated from subsequent analyses.

Previous reports on the use of PyMS as a taxonomic technique among microorganisms have suggested that phenotypic variation within samples influences the accuracy of the method (Freeman *et al.*, 1994). The results presented here are not entirely consistent with this finding. PyMS analysis of species from within a single shore population showed that species could be separated into discrete groups (Figs. 4.3 – 4.5). Given the amount of environmental 'noise', for example from epiphytes and other contaminants, to be expected in a shore habitat this result is encouraging. Comparisons among species between shores showed some discrepancies between populations. However, analysis of similarity (ANOSIM) carried out on between-shore groups of species showed that geographical location was not a significant factor in discrimination among similar species types. There were some exceptions to this finding. Firstly, among shores 1 and 2,

the *A. nodosum* groups did not form a discrete cluster. Confusingly, when shores 1, 2 and 3 were compared this was no longer the case and the *A. nodosum* groups did form a discrete cluster (Fig. 4.8). *A. nodosum* is often observed to be covered with growth of the epiphytic alga *Polysiphonia lanosa* (Norton, 1985); although care was taken to avoid contamination of the samples, it is possible that this potential source of variation was not completely eliminated and may also account for mis-identification by PyMS. In PyMS analyses, PC-CV examines the within-group variability as compared to the between-group variability of the data set. This may account for the re-grouping of *A. nodosum* compared among three shores (Fig. 4.8). Sampling artefacts in the variability of five individuals could also be a further source of error.

The similarity dendrogram derived for shores 1, 2 and 3 (Fig. 4.8) shows within-type congruence across all shores among *A. nodosum* replicates, and also across all shores among *F. serratus* replicates. These two species appear as clearly distinct from all other species. However, incomplete separation was achieved for *F. vesiculosus* and *F. spiralis* populations. The occurrence of hybrids of *F. spiralis* and *F. vesiculosus* plants has been widely reported for natural populations (Burrows & Lodge, 1954; Powell, 1963; McLachlan *et al.*, 1971; Scott & Hardy, 1994; Mulyadi & Hardy, 1996). This may account in part for the incomplete separation of these two species in the similarity dendrograms derived for species collected at shores 1, 2 and 3, suggesting that some introgression between these species has indeed occurred at these sites.

The grouping of *A. nodosum* and *A. nodosum mackaii* into two discrete clusters (Figs. 4.9 – 4.10) illustrates the ability of PyMS to detect differences at the sub-species level. The recognition of within-species differences in PyMS has already been demonstrated by analysis of microorganisms (Goodfellow *et al.*, 1997), demonstrating that the discriminatory ability of PyMS was sufficient to detect very small genotypic changes in, for example, bacteria (Goodacre

& Berkeley, 1990). Russell (1995) cultured *Pilayella littoralis* isolated from two geographically distinct locations, namely the Baltic and Irish Seas, under identical growth-room conditions. These two isolates remained separable in PyMS, which he concluded proved their status as separated subspecies. The underlying genetic variation implied by such work is clearly detectable by PyMS. Since the difference among similar species types collected from separate shores was shown to be of no statistical significance in this study, it was concluded that PyMS confirmed the ecotype status of *A. nodosum mackaii*.

Figure 4.8 illustrates the similarity dendrogram derived for PyMS analysis of representatives of the Order Fucales. The Fucaceae were initially described as a monophyletic group with a divergence between monoecious and dioecious species (Powell, 1963). Serrão *et al.* (1999) investigated the evolutionary relationships among the Fucaceae based on sequences of the internal transcribed spacer region of nuclear ribosomal DNA obtained from 16 species representing all genera within the order. Their work confirmed the monophyletic origin of the Fucaceae but suggested that divergence between species was not based on monoecy / dioecy. PyMS analysis showed agreement with the phylogenetic relationships in the Fucaceae reported by Serrão *et al.* (1999) rather than the well established model for this group proposed by Powell (1963). PyMS analysis showed that all species of *Fucus* were closely related, with the exception of *F. ceranoides*. *F. ceranoides*, an estuarine species, appears in the PyMS-derived dendrogram to have more similarities with *Pelvetia canaliculata* than any other species within the Fucaceae. This is a confusing result that cannot be easily explained other than that it possibly shows a measure of phenotypic, and perhaps environmental similarity (since *Pelvetia* is a high shore inhabiting species).

The PyMS dendrogram placed *Halidrys siliquosa* (Cystoseiraceae) as a sister clade to the Fucaceae, more similar than *Himanthalia elongata* (Himanthaliaceae), which was placed among the Cystoseiraceae-Sargassaceae cluster. This result does not agree with the phylogeny reported for the Fucales by Rousseau and de Reviers (1999), which placed the Himanthaliaceae as a sister clade to the Fucaceae and *Halidrys* among the Cystoseiraceae. The phylogenetic position of *Himanthalia* has not been completely resolved by molecular investigations of the Fucales (Rousseau *et al*, 1997; Rousseau and de Reviers, 1999) but has been grouped with the Fucaceae clade. *Himanthalia* is morphologically very dissimilar to all the other Fucales, which may account for the placement of this species as a separate branch in the PyMS-derived dendrogram.

The Cystoseiraceae have been described as polyphyletic and closely related to the Sargassaceae (Rousseau & de Reviers, 1999). This appears to be the pattern emerging from PyMS analysis, but with the small number of samples it is difficult to draw any firm conclusions. The relative positions of *Sargassum* and *Cystoseira* species, which are morphologically very similar, within the Cystoseiraceae-Sargassaceae group also remain controversial (Rousseau *et al*., 1997) and the merging of these two families has been proposed (Saunders & Kraft, 1995).

It may be concluded that PyMS does not provide satisfactory information on the evolutionary relationships amongst the Fucales, but that it does provide a measure of the phenotypic and, to some extent, underlying genetic information of these species. Consequently, the results achieved through PyMS analysis should be treated with caution and interpreted to greatest advantage in combination with other investigation of the subject material. PyMS may be of value as a tool for the preliminary study of a controversial issue, for example where phylogenetic relationships are questionable such as exist within the Cystoseiraceae-Sargassaceae group.

PyMS can only contribute to the complex subject of taxonomy as a measure of chemical phenotype. Since it may be argued that all taxonomic decisions are subjective, it is imperative that such decisions be based on as much information as possible. Systematic studies in which PyMS is coupled with classical morphological and physiological studies will certainly contribute to better understanding of the attributes of organisms, especially those which were previously difficult or impossible to study.

Taxonomic inconsistencies and the uncertainty of dealing with intraspecific variation are still unresolved problems in algal systematics. As a chemotaxonomic technique, PyMS has advantages in that it is rapid, direct and requires very small amounts of material. The inclusion of a larger sample size from as many different sites as possible would undoubtedly improve the accuracy and confidence that may be placed in PyMS results. The investigation of seasonal and temporal differences, detectable by PyMS, among samples would also be of interest.

Chapter 5

Evaluation of the Discriminatory Capacity of PyMS with respect to Variation in Environmental Condition II: Laboratory Studies of *Skeletonema costatum*

5.1 Introduction

Marine phytoplankton contribute significantly to primary productivity, which is known to be nutrient limited in many areas of the ocean (Dugdale, 1967; Officer & Ryther, 1980). However, aspects of the ecology and physiology of marine phytoplankton, including the influence of environmental conditions such as nutrient depletion and light deprivation, are poorly understood (Berges & Falkowski, 1998). The marine diatom *Skeletonema costatum* (Grev.) Cleve. (Bacillariophyceae), which is a common component of the phytoplankton in coastal and estuarine areas from late spring to summer (Hasle & Smayda, 1960), was chosen for this study. *S. costatum* exhibits great ecotypic diversity (Gallagher, 1982) as well as phenotypic plasticity (Gallagher *et al.*, 1984); it is also ecologically important and has been recorded as a key species in the formation of harmful algal blooms in Japan (Yamomoto & Tsuchiya, 1995).

Nutrient levels and ratios are affected by hydrographic and biogeochemical processes (Watts *et al.*, 1998) as well as a range of environmental variables including, temperature (Paasche, 1980), light intensity (Davis, 1976) and biogenic production (Brzezinski, 1985). Nutrient limitation has been shown to have a particular effect on diatom growth rate and morphology (Busby & Lewin, 1967; Davis *et al.*, 1973; Harrison *et al.*, 1977; Yamomoto & Tschuchiya, 1995). For example, nitrogen limitation has been shown to directly affect the ability of cells to synthesise proteins (Berges & Falkowski, 1996), and limitation of silica, which is required by diatoms for the formation of their exoskeleton, has been demonstrated to cause changes in morphology (Yamomoto & Tschuchiya, 1995). Under conditions of environmental stress, including nutrient depletion, diatoms have been reported to employ survival strategies, such as the formation of resting cysts, a process known as 'sinking' (Harris, 1995).

The influence of salinity variation on diatom growth has also been investigated; for example, a salinity decrease resulted in an increase in the cellular silica content for experimental species and was thought to be a response to osmotic stress (Vrieling *et al.*, 1999). In coastal waters salinity increases as a function of depth (Grantham & Tett, 1993; Watts *et al.*, 1998). Silicate concentration varies inversely with salinity and is highest in the surface layers, probably due to the influence of river run-off (Watts *et al.*, 1998).

Light deprivation has also been shown to limit the ability of diatom cells to assimilate nutrients required for metabolic functions and new cell synthesis (Berges & Falkowski, 1998). Previous studies also demonstrated that all major phytoplankton groups (diatoms, dinoflagellates, green algae and cyanobacteria) altered their cellular pigment content, and exhibited differences in growth rates, in response to changes in growth light-intensity (Beardall & Morris, 1976; Gallagher *et al.*, 1984; Gallagher & Alberte, 1985). Furthermore, low light intensity increases the Si:N ratios of S. costatum (Brzezinski, 1985). Photosynthetically active radiation (irradiance) decreases with increasing depth (Watts *et al.*, 1999).

The influence of environmental growth conditions on bacterial composition can be detected by pyrolysis mass spectrometry (Boon *et al.*, 1981; Freeman *et al.*, 1994b). PyMS has also been used previously to establish temporary classifications for environmental isolates of organisms, for example bacteria, which were difficult to identify by conventional methods (Helyer *et al.*, 1993). The potential of using PyMS as a rapid, highly discriminatory technique for the characterisation of large numbers of natural isolates was illustrated by such studies (Helyer *et al.*, 1993).

Chapter 4 examined PyMS data for macroalgae, sampled directly from the environment, which were expected to exhibit natural variation in phenotype in response to differences in habitat. The simple laboratory investigations described here were carried out using axenic cultures of microalgae, which were grown under environmentally controlled conditions. *S. costatum* is easy to culture in the laboratory, and literature on field observations and laboratory experiments that relate to this species is also extensive (Brunet, et al., 1996; Sanchez-Saavedra & Voltolina, 1996; Smayda, 1997; Kunnis, 1998; Geider *et al.*, 1998; Balode *et al.*, 1998).

The main objectives for this study were to manipulate growth conditions for cultures of one axenic clone of *S. costatum* and to examine any resulting variation in pyrolysis mass spectra between cultures. Three main environmental regimes, which attempted to simulate conditions that could be expected in the natural environment, were imposed upon the diatom cultures. These were, low salinity, limited silicate and low irradiance. Previous analyses by PyMS of *S. costatum* or any other diatom cultured under variable conditions have not been reported.

5.2 Materials and Methods

5.2.1 Diatom strain and culture conditions

Axenic cultures of the diatom *Skeletonema costatum* (Grev.) (strain CCAP 1077/5) were used in the experiments reported here. Unless otherwise stated, batch cultures of *S. costatum* were maintained in 'F/2 +Si' media (Guillard & Ryther, 1962) (Appendix 1), at a salinity of 32 practical salinity units (PSU) and under continuous fluorescent illumination of 150μE m^{-2} s^{-1} and 15° C. Silicate ($Si(OH)_4$) was present in 'F/2 + Si' media at a concentration of 40 μM. All media were made using artificial seawater (Waterscene Scientific Marine Salts) and had a final pH of 8.0. Aliquots of 250ml of *S. costatum* were cultured in 500ml glass flasks, which were gently shaken once a day throughout the experimental period. All glassware was washed in 10%

hydrochloric acid and media were autoclaved at 121 °C for 15 minutes prior to use. All culture transfers were carried out aseptically and the absence of bacteria was verified by microscopic examination of cultures at x 400 magnification. Sub-culturing was carried out at two-week intervals in order to maintain *S. costatum* throughout the experimental period. Cultures were grown in the appropriate environmental regime prior to experimentation.

5.2.2 Growth measurements

Five millilitre aliquots of inocula were collected daily from each of 3 replicate flasks for cell counts. The same flasks were used throughout and sampling was carried out without replacement. Cell concentrations were calculated using a Neubauer haemocytometer slide over an eight-day period. Batches of 25 x 0.25 mm^2 squares on the haemocytometer were counted in triplicate for each sample.

5.2.3 Experimental conditions

Experiments were started with a volumetric addition of inocula (Table 5.1 a-c) sampled from exponential growth phase of the culture stock and were allowed to proceed for 6 days. Cells were harvested during exponential growth phase and concentrated by centrifugation at 20°C and 5000G for 10 minutes before being stored at –20°C in eppendorf tubes until analysis. All experiments were carried out in replicate batch culture and maintained under the light and temperature regime described above. Experimental conditions are summarised in Tables 5.1 – 5.3 for each of the following experimental procedures.

(I) Culture of *Skeletonema costatum* in different salinities

Growth of *S. costatum* in media at four salinities (32 PSU, 30 PSU, 25 PSU and 20 PSU) was investigated. All other constituents of the growth media were added at 'F/2 + Si' concentrations

(Table 5.1). Five replicates of 250 ml of each media, in 500ml flasks capped with aluminium foil, were inoculated with 10ml of *S. costatum*.

(II) Culture of *Skeletonema costatum* in different silicate ($Si(OH)_4$) and salinity concentrations

Growth of *S. costatum* under varying salinity and silicate regimes was investigated. Salinity and silicate concentrations of three media were based on field observations made for seawater nutrient profiles in a Scottish sea-loch during a spring phytoplankton bloom (Watts *et al.*, 1998) and are summarised in Table 5.2. All other nutrients were maintained at normal 'F/2 + Si' concentrations. Cultures were also grown in 'F/2 + Si' medium at a salinity of 32 PSU for this experiment. Five replicates of 250 ml of each media, in 500ml flasks capped with aluminium foil, were inoculated with 10ml of *S. costatum*.

(III) Culture of *Skeletonema costatum* under different light conditions

Blue and neutral density filters were used to simulate four photosynthetically active radiation (PAR) levels normally encountered throughout the photic zone (Watts *et al.*, 1999). These were 150μE$m^{-2}s^{-1}$, 100μE$m^{-2}s^{-1}$, 50μE $m^{-2}s^{-1}$ and 15μE $m^{-2}s^{-1}$. Five replicates of 10ml of 'F/2 + Si' medium at 32 PSU were inoculated with 1ml of *S. costatum* culture and grown under each light regime in 20ml test tubes capped with aluminium foil. Cultures were maintained under continuous light and at 15°C (Table 5.3).

5.2.4 Sample preparation for pyrolysis mass spectrometry

Samples were defrosted immediately prior to PyMS analysis and 5 μl of the diatom concentrate was applied to clean 530 °C curie-point foils. Five microlitre samples of the media used for each experiment were also analysed in the same batch run to determine whether any differences between samples was due to growth and not just an artefact of differences between the media.

Six replicates from each sample were prepared and analysis then proceeded as described in sections 2.1.2-2.1.4.

Table 5.1
Media and growth conditions for experiment I.

Salinity (practical salinity units)	Silicate ($Si(OH)_4$)	Volume of media (ml)	Initial volume of inoculum (ml)	Volume of culture vessel (ml)	Culture Conditions (irradiance, temp.)
32	F/2 +Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
30	F/2 +Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
25	F/2 +Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
20	F/2 +Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C

Table 5.2
Media and growth conditions for experiment II.

Salinity (practical salinity units)	Silicate ($Si(OH)_4$)	Volume of media (ml)	Initial volume of inoculum (ml)	Volume of culture vessel (ml)	Culture Conditions (irradiance, temp.)
32	F/2 +Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
30	F/2 + 6 µM Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
25	F/2 + 8 µM Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
20	F/2 + 9 µM Si	250	10	500	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C

Table 5.3
Media and growth conditions for experiment II.

Salinity (practical salinity units)	Silicate ($Si(OH)_4$)	Volume of media (ml)	Initial volume of inoculum (ml)	Volume of culture vessel (ml)	Culture Conditions (irradiance, temp.)
32	F/2 +Si	10	1	20	150 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
32	F/2 + Si	10	1	20	100 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
32	F/2 + Si	10	1	20	50 µE $m^{-2}s^{-1}$ (24 hrs), 15°C
32	F/2 + Si	10	1	20	15 µE $m^{-2}s^{-1}$ (24 hrs), 15°C

5.2.5 Data analysis

Pre-processing of sample data was carried out as described in section 2.2.1 followed by principal components analysis (PCA) and canonical variates analysis (CVA). ANCOVA were performed

on specific growth rate data with day as the co-variate, in Minitab (Minitab Inc., 1998), using a general linear model (GLM), to determine any significance of variation in *S. costatum* growth over time under different experimental conditions; data were Arcsin-square-root transformed. Two-way crossed ANOSIM were performed on *S. costatum* PyMS data to determine any significance of experimental treatments on group discrimination. ANOSIM analyses were carried out using the PRIMER v4.0 software package (Plymouth Marine Laboratory, NERC, 1994).

5.3 Results

5.3.1 Growth

Cell growth varied over time at different salinities (Figure 5.1 a), and Si $(OH)_4$ concentrations (Figure 5.1 b). There were significant differences, shown by ANCOVA (General Linear Model), in growth of *S. costatum* among media at different salinities ($F=88.16$, $p<0.0001$) and among media at different $Si(OH)_4$ concentrations ($F=192.38$, $p<0.0001$).

5.3.2 Discrimination between *Skeletonema costatum* grown under different conditions

In all experimental treatments there were significant differences in *S. costatum* PyMS data among the media in which specific cultures were grown (Table 5.2).

(I) Salinity

There were differences in pyrolysis mass spectra among all salinity treatments of statistical significance (ANOSIM, $R=0.534$, $p< 0.0001$) (Fig. 5.2, Table 5.3). PC-CV analysis discriminated between *S. costatum* cultured in the '32 PSU' medium and all other groups; discrimination among the three treatment media at '30 PSU', '25 PSU' and '20 PSU' was less well-defined (Fig. 5.2).

(II) Silicate and salinity

PC-CV analysis of pyrolysis mass spectral data showed overall discrimination between groups of *S. costatum* grown in treatment media at different silicate and salinity concentrations (ANOSIM, R=0.539, P<0.0001) (Fig. 5.3). All treatments groups were statistically different from each other except for *S. costatum* grown in the '30 PSU + 6 μM Si' and '20 PSU + 9μM Si' (Table 5.4).

(III) Irradiance

There were overall differences in pyrolysis mass spectra among all irradiances (ANOSIM, R=0.571, p<0.0001) and between each treatment group (Table 5.5). PC-CV analysis of pyrolysis mass spectra for *S. costatum* grown under the four different light regimes showed two well-defined clusters corresponding to the groups grown under the two highest irradiances and those grown under the two lowest irradiances (Fig. 5.4). PC-CV analysis also showed mis-identification by PyMS of one replicate from each of the *S. costatum* samples cultured under '33 μE m^{-2} s^{-1}' and '10 μE m^{-2} s^{-1}' (Fig. 5.4).

Table 5.2
ANOSIM results for differences in *S. costatum* cultures and growth media

Factor	Global R	p
Media and *S. costatum* expt. (I)	0.947	< 0.0001
Media and *S. costatum* expt. (II)	0.756	< 0.0001
Media and *S. costatum* expt. (III)	1.0	< 0.0001

Table 5.3
Pairwise ANOSIM results for salinity

Factor	Global R	p
32 PSU, 30 PSU	0.47	< 0.008
32 PSU, 25 PSU	0.72	< 0.001
32 PSU, 20 PSU	0.89	< 0.001
30 PSU, 25 PSU	0.31	< 0.017
30 PSU, 20 PSU	0.78	< 0.001
25 PSU, 20 PSU	0.69	< 0.001

Table 5.3
Pairwise ANOSIM results for salinity

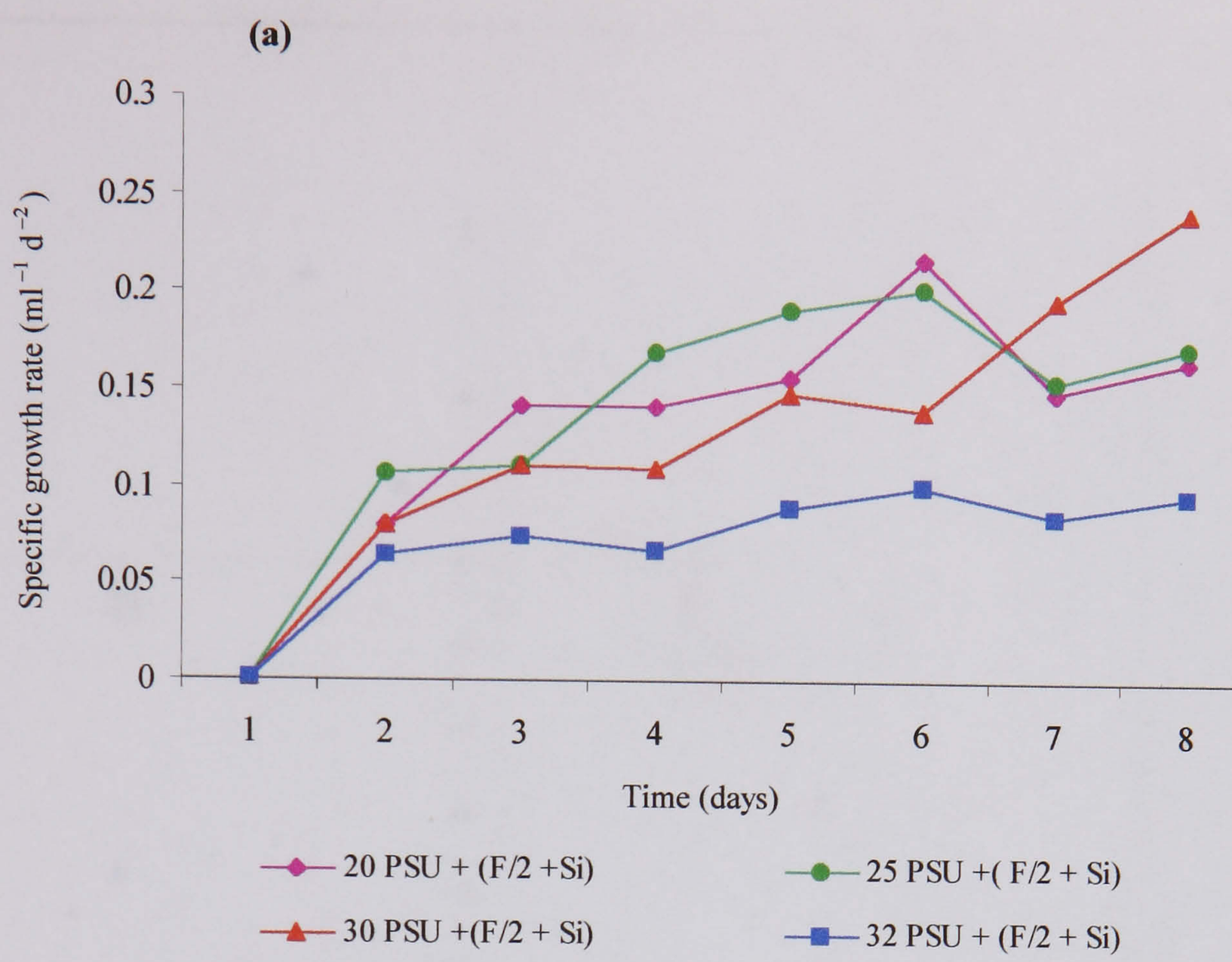
Factor	Global R	p
32 PSU, 30 PSU	0.47	< 0.008
32 PSU, 25 PSU	0.72	< 0.001
32 PSU, 20 PSU	0.89	< 0.001
30 PSU, 25 PSU	0.31	< 0.017
30 PSU, 20 PSU	0.78	< 0.001
25 PSU, 20 PSU	0.69	< 0.001

Table 5.4
Pairwise test results from two-way ANOSIM results among silicate

Factor	Global R	p
32 PSU + (F/2 +Si), 30 PSU + (6 μM Si $(OH)_4$)	0.42	< 0.006
32 PSU + (F/2 +Si), 25 PSU (8 μM Si $(OH)_4$)	0.77	< 0.002
32 PSU + (F/2 +Si), 20 PSU (9 μM Si $(OH)_4$)	0.67	< 0.002
30 PSU + (6 μM Si $(OH)_4$), 25 PSU (8 μM Si $(OH)_4$)	0.35	< 0.019
30 PSU + (6 μM Si $(OH)_4$), 20 PSU (9 μM Si $(OH)_4$)	0.26	= 0.062
25 PSU (8 μM Si $(OH)_4$), 20 PSU (9 μM Si $(OH)_4$)	0.53	< 0.002

Table 5.5
Pairwise ANOSIM results for irradiance

Factor	Global R	p
100 μE m^{-2} s^{-1}, 66 μE m^{-2} s^{-1}	0.64	< 0.0001
100 μE m^{-2} s^{-1}, 33 μE m^{-2} s^{-1}	0.70	< 0.0001
100 μE m^{-2} s^{-1}, 10 μE m^{-2} s^{-1}	0.69	< 0.001
66 μE m^{-2} s^{-1}, 33 μE m^{-2} s^{-1}	0.45	< 0.001
66 μE m^{-2} s^{-1}, 10 μE m^{-2} s^{-1}	0.39	< 0.002
33 μE m^{-2} s^{-1}, 10 μE m^{-2} s^{-1}	0.78	< 0.0001



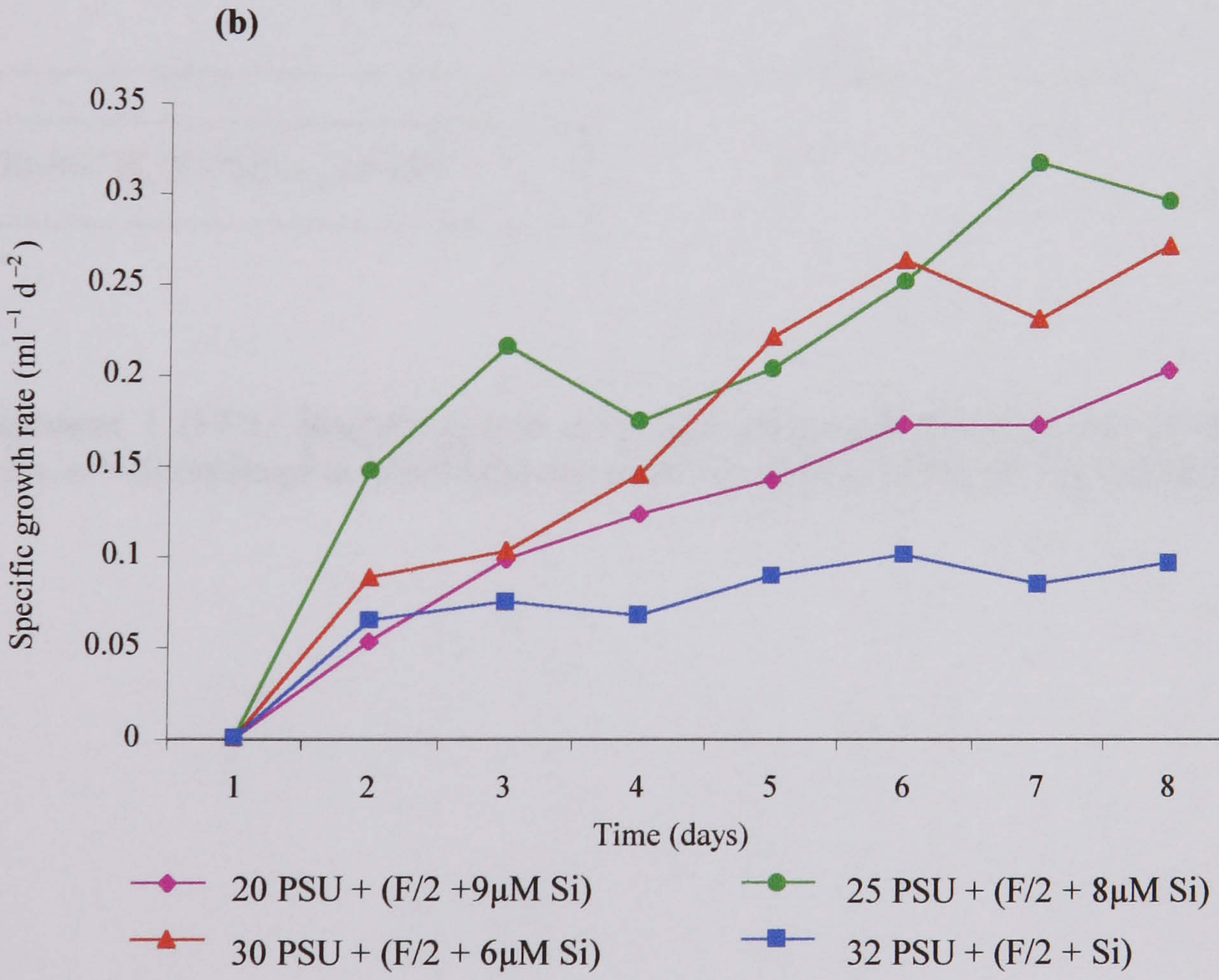


Figure 5.1 (a – b)
Specific growth rate (compared to day 1) of *Skeletonema costatum* against time as a function of (a) variation in salinity (F/2 +Si concentration of all nutrients) and (b) variation in $Si(OH)_4$ concentration in media of varying salinity.

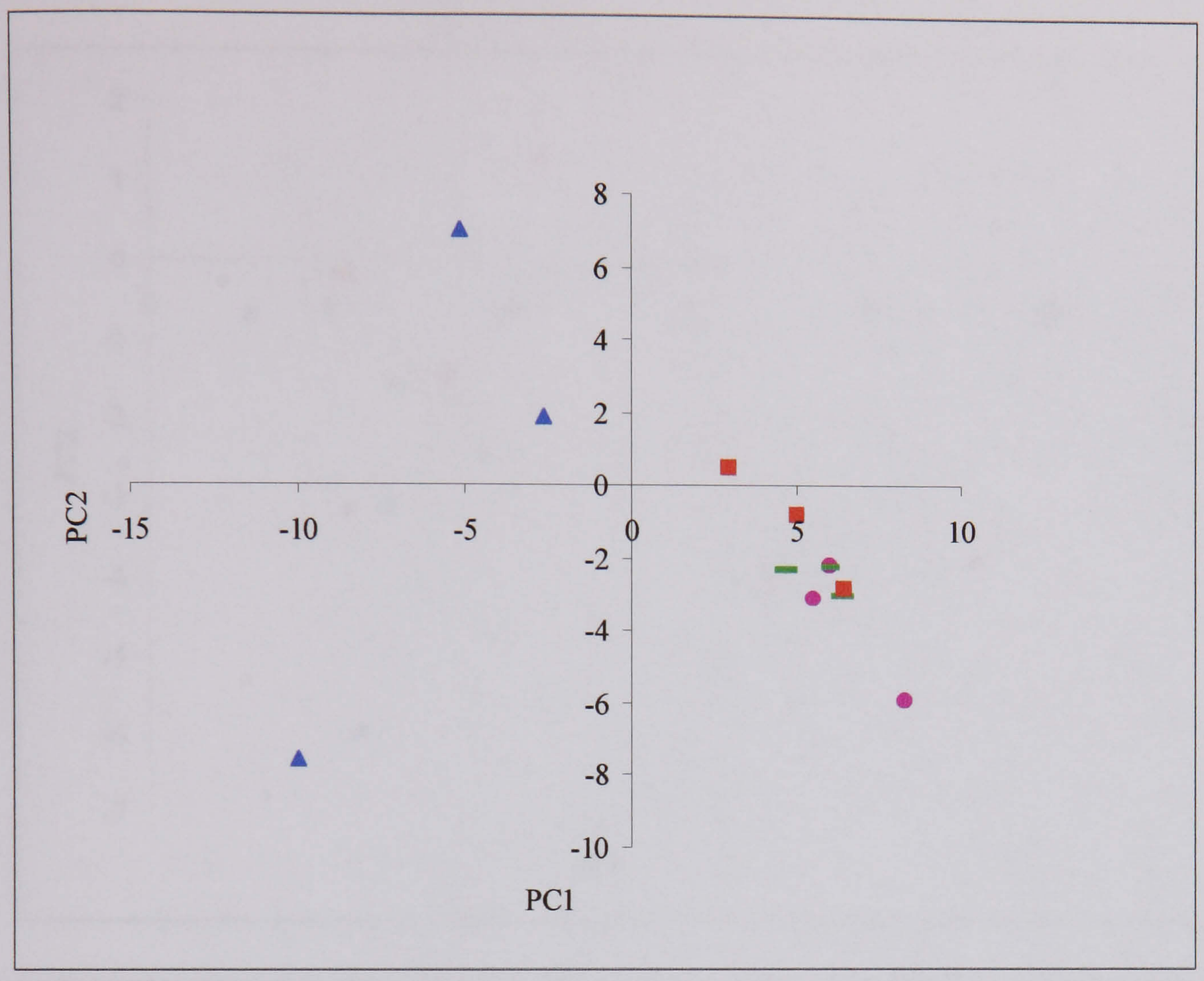


Figure 5.2
Principal component 1 (PC1) plotted against principal component 2 (PC2) for PyMS data of *S. costatum* (means, n = 3) cultured in 4 different media with salinities of 20, 25, 30 and 32 PSU.

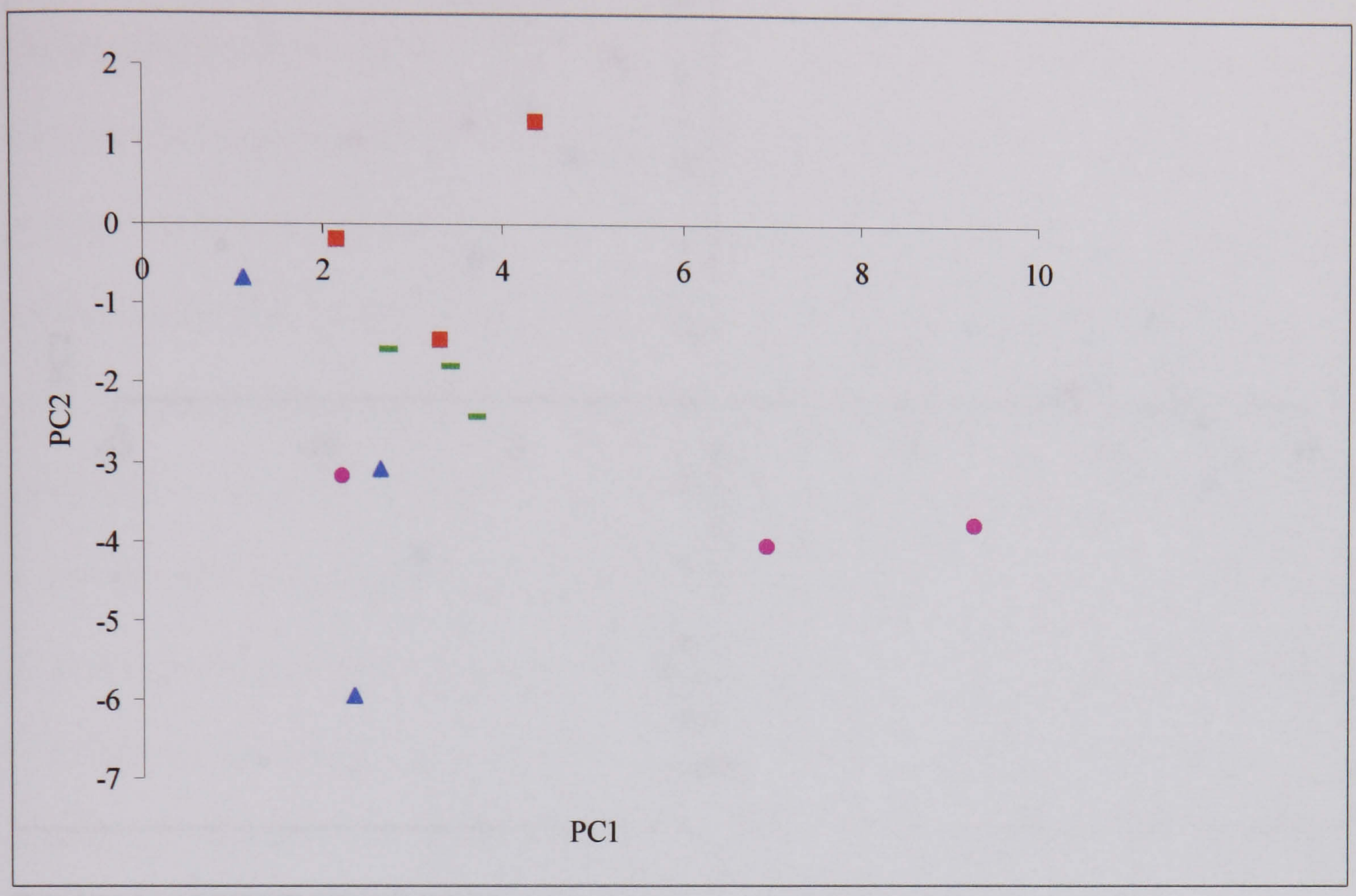


Figure 5.3
Principal component 1 (PC1) plotted against principal component 2 (PC2) for PyMS data of *S. costatum* (means, n = 3) cultured in 4 media with different salinity and $Si(OH)_4$ concentrations.

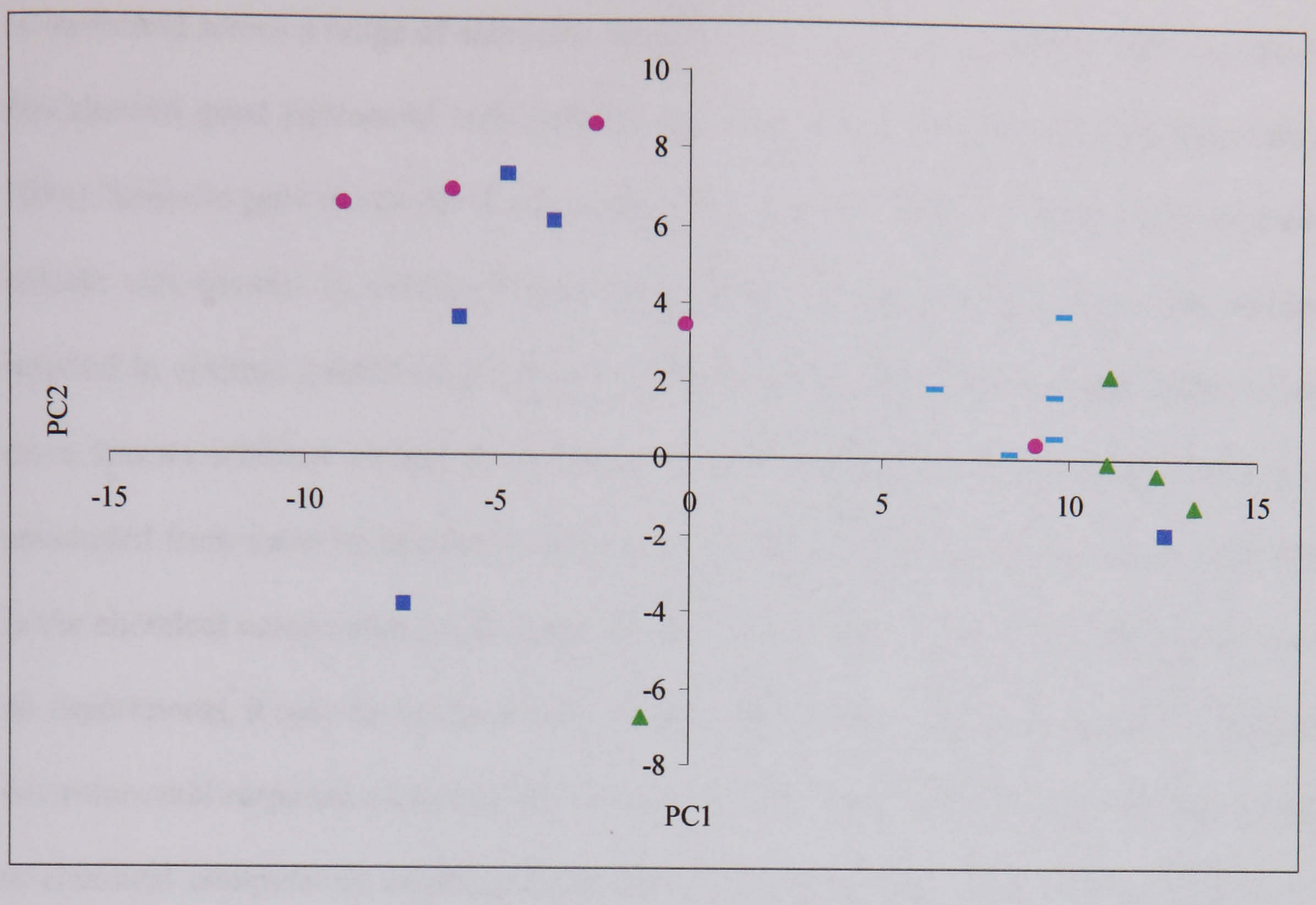


Figure 5.4
Principal component 1 (PC1) plotted against principal component 2 (PC2) for PyMS data of *S. costatum* (means, n = 3) cultured in 'F/2 +Si' medium under 4 different irradiance regimes.

5.4 Discussion

PyMS clearly has potential as a rapid technique for the analysis of microalgae, and also for the detection of small variations among samples due to changes in environmental conditions.

Chemical differences between media did not affect the sample groupings for cultures of *S. costatum* (Table 5.2), implying that statistically significant differences in pyrolysis mass spectra among cultures were due to specific differences among *S. costatum* as opposed to the media themselves. Specific growth rates of *S. costatum* were also significantly different among

different salinities and different silicate concentrations (Fig. 5.1 a – b). That viable growth could be sustained across a range of salinities, from 20 PSU to 32 PSU, was illustrated by these results; this showed good agreement with salinity responses of *S. costatum* reported elsewhere (Brand, 1984). Specific growth rate for *S. costatum* was lowest in '32 PSU + (F/2 + Si)' medium, where silicate was present in excess. It was not possible to determine which of the salinity media resulted in optimal growth (Fig 5.1 a) from the experiments conducted here, however pyrolysis mass spectra differed among *S. costatum* cultures in different salinities (Fig 5.2). It may be concluded from these results that variation in growth rate among cultures resulted in differences in the chemical composition of *S. costatum* cells. Given that the same axenic species was used in all experiments, it may be further concluded that these differences were directly attributable to an environmental response expressed by *S. costatum*. That PyMS may be used to detect differences in chemical composition resulting from differences in growth rate has been reported for other organisms (Nieman et al., 1992).

Groups of *S. costatum* grown in media with different silicate concentrations were shown to be significantly different in PyMS analysis (ANOSIM, $R=0.539$, $P<0.0001$) (Fig. 5.3). Among the three media with limited silicate concentration, growth rate appeared to be highest in the '25 PSU + (F/2 + 8 μM Si)' medium, followed by the '30 PSU + (F/2 + 6 μM Si)' and the '20 PSU + (F/2 + 9 μM Si)' in that order (Fig. 5.1 b). The sensitivity of diatoms to silicate limitation may be of importance in the adaptive 'sinking' response exhibited under conditions of environmental stress (Harris, 1995; Harrison *et al.*, 1986). Sinking in diatoms is accomplished by changes in the metabolic activity of the cell (Bienfang *et al.*, 1982). A faster increase in growth rate in the media with low silicate concentration in the present experiments may reflect the ability of *S. costatum* to adapt its metabolic activity to silicate-limited conditions. Other reports have also suggested that *S. costatum* is capable of responding physiologically to its environment, for

example, by changing its sinking rate between nutrient-rich and nutrient-depleted conditions (Bienfang *et al.*, 1982). The present investigation of the effect of silicate limitation on *S. costatum* (experiment II) also showed agreement with previous growth rate studies on *S. costatum* (Yamomoto & Tsuchiya 1995). Higher growth rates were observed for *S. costatum* supplied with silicate with an accompanying salinity decrease than without a salinity decrease. Yamomoto and Tsuchiya (1995) concluded that silicate-depleted *S. costatum* grew faster in situ if salinity was optimum. Furthermore, silicate limitation caused several distinct morphological changes in *S. costatum* such as cell elongation (Yamomoto & Tsuchiya, 1995). Therefore, although morphology was not examined in the present study, significant differences in pyrolysis mass spectra of *S. costatum* cultures in media with different silicate concentrations may be linked to morphological variation among samples. PyMS-derived differences may also be attributed to chemical variations among samples due to differences in the ratios of cellular components. PyMS detection of differential chemical allocation of cellular components has previously been demonstrated for tomato variants (Nieman *et al.*, 1993). Furthermore, Sahota *et al.* (1992) reported the use of Py-GC/MS as a method for measuring cellular DNA levels and concluded that this technique could also be applicable to determine quantities of other cellular constituents in complex biological systems. More detailed investigations of factors that influence growth, such as nutrient uptake rates, would be necessary to form any firm conclusions about the variations in growth rates recorded here.

Pyrolysis mass spectra of *S. costatum* grown under the different light regimes used in this investigation showed significant differences among groups (ANOSIM, $R=0.571$, $p<0.0001$). PC-CV analysis showed two well-defined clusters corresponding to the groups grown under the two highest irradiances and those grown under the two lowest irradiances (Fig. 5.4). Brzezinski (1985) reported that low light intensity increased the ratio of silicon to both carbon and nitrogen

in marine diatoms, resulting in a higher cellular content of silicon. Furthermore, low light intensity has been demonstrated to result in an increase in the Si:N ratio of nitrogen-limited *S. costatum* back toward nutrient-enriched values (Davis, 1976), showing that this species is capable of exhibiting an adaptive response to its environment. Culver and Smith (1989) proposed that variations in the environment which affect physiological processes cause a change in sinking rates. Previous investigations of light-limited growth in diatoms also showed significant changes in cell volume (Berges & Harrison, 1993) and increases in cell concentrations of photosynthetic pigments (Gallagher & Alberte, 1985). Variation in cell volume was thought to be a significant metabolic adaptation, necessary for maintenance of correct cellular concentrations of metabolites and catalysts (Raven, 1981). Hence PyMS differences among spectra for *S. costatum*, detected in the irradiance experiment reported here, may reflect either physiological or morphological, owing to changes in cellular silicate concentration, variations between the cultures, or a combination of the two. These results were congruent with the findings of Freeman *et al.* (1994b) who showed that PyMS could detect phenotypic variation among bacteria, as a result of growth. The potential for PyMS to detect compositional differences between cells grown in different environmental conditions was demonstrated by Magee *et al.* (1997b) based on temperature-dependent compositional shifts in a *Pseudomonas* species.

The experiments described above were very simple, and considered only one clone of one diatom species. However given the complex nature of interacting factors affecting growth of such organisms, it was considered to be the best approach to an initial and novel use of PyMS. The precise nature of implied chemical differences in *S. costatum* could not be determined. However, the results presented here suggest that PyMS may be of value in discriminating between environmental isolates of microalgae, or the detection of intraspecific variability, prior to the use of other techniques which may be more expensive in terms of time or consumables.

PyMS does not allow absolute quantitation and is not a substitute for biochemical assays designed to provide detailed compositional information on a restricted compound class, but complements existing techniques.

The use of PyMS as a tool for providing temporary identifications of samples, which are analysed along with known reference strains ('operational fingerprinting'), has been well documented (e.g., Meuzelaar *et al.*, 1982; Gutteridge *et al.*, 1985). The use of 'operational fingerprinting' as a complementary tool to the 'autoecological approach' to phytoplankton ecology may be of interest for future studies of this nature.

Chapter 6

General Discussion

6.1 The potential value of PyMS

Curie-point pyrolysis mass spectrometry (PyMS) is a highly discriminatory technique that analyses chemical characteristics of organic material. It has been successfully applied to a number of studies in bacterial systematics but its use for the investigation of multi-cellular organisms has been limited. The full extent of its applicability to the analysis of biological material has not yet been realised. PyMS considers total cellular composition of the sample material and has proved to be sufficiently sensitive to detect differences at the sub-species level (Sisson *et al.*, 1991). The intrinsic value of this method lies in its ability to resolve chemical phenotype as well as reflecting inherent genetic mechanisms and has not been fully explored in previous studies.

This study aimed to investigate the potential applications and limitations of PyMS as an analytical technique for marine organisms by investigation of biological and chemical variation among macro- and micro- algae. Four specific investigations were central to this work:

- Assessment of the technical constraints of the PyMS method for use in the routine analysis of simple organic material.
- Determination of the effect of sample concentration on pyrolysis mass spectral data.
- Evaluation of the robustness of the PyMS method in relation to environmentally induced variation in field populations of macroalgae from the order Fucales.
- Determination of the effect of experimentally induced variation in laboratory cultures of the marine diatom *Skeletonema costatum* on pyrolysis mass spectral data.

6.1.1 Technical constraints

Lack of reproducibility has been identified as a limiting factor in the use of PyMS as a routine analytical technique. However PyMS offers some advantages over other biochemical methods in

that it is rapid, relatively inexpensive and allows the analysis of a large number of samples in a single batch run. To what extent the technical constraints of the PyMS method limit its use for wide application to biological systems is an important consideration. This study aimed to assess the usefulness of this technique, firstly by experimental investigation of the variability of the pyrolysis mass spectra produced for a series of simple organic materials. This specifically involved the investigation of day to day variability and the effect of sample concentration on pyrolysis mass spectral data.

In chapter 3, sample concentration was shown to have a distinct effect on PyMS data and group discrimination. Total ion count, which is a direct measure of the amount of sample pyrolysed, was correlated with sample concentration (Fig. 3.2 a-d). The results from this study show congruence with other investigations which recommended that a minimum total ion count of 5 x 10^5 was required for PyMS analyses to produce meaningful results (Freeman *et al.*, 1995). Although sample size required for PyMS analysis is small (~ 5 µl), there is clearly an effect of sample concentration which must be taken into consideration for any analysis. Currently accepted PyMS methodology does not include recommendations for determination of sample concentration prior to pyrolysis and achieving a suitable ion count has largely been a process of trial and error (e.g. Mathers *et al.*, 1997). However, recommendations for routine instrumental maintenance include the periodic analysis of a 60 µM solution of glycogen, which produces a characteristic and reproducible pyrogram, as a check on instrumental drift (Horizon Instruments, 1988). This clearly emphasises the need for the use of standardised sample concentrations in the routine use of PyMS.

The discriminatory ability of PyMS was illustrated by the clear distinction of chemically similar amino acid solutions at concentrations ranging from 60 µM to 0.6 µM (Figs. 3.6 - 3.9). Pyrolysis

mass spectra for solutions of each of glycogen, alanine, lysine, glycine and phenylalanine were significantly different among batch runs (ANOSIM R=0.72, p<0.0001), however, group membership was unaffected (Table 3.5 a - b). The conservation of correct group membership over a 28-day experimental period in this study is congruent with the findings of previous investigations (Manchester & Goodacre, 1995). PyMS-derived cluster analysis discriminates among samples by comparing and contrasting the within-group variability to the between-group variability. Once the day to day variability approaches that of the between-group variability it must be concluded that PyMS is no longer capable of providing useful data.

6.1.2 Environmental variability I – Field studies on macroalgae

PyMS analyses of macroalgal species from the order Fucales, presented in chapter 4, challenge the assertion that PyMS is of value as phenotypic technique only. There were overall similarities among representatives of species sampled from both within one shore (Figs.4.3 – 4.5), and from different shores (Figs. 4.7 – 4.8). PyMS-derived similarity dendrograms for the species analysed in this study also showed clustering congruent with accepted phylogenies for this order (Fig. 4.10). Differences were shown among different tissues of *Himanthalia elongata* (Fig. 4.6), suggesting that chemical characterisation of the specific physiology or function of cells may be traced by PyMS. Nieman *et al* (1990) characterised different tissues from stems of young and old carnation plants by pyrolysis-gas chromatography/mass spectrometry and concluded that this technique could be used to provide information on the chemical nature of cell walls.

Differences were also shown between the species *Ascophyllum nodosum* and the ecotype *A. nodosum mackaii* (Fig. 4.9) suggesting that PyMS resolves chemical information on organisms at the sub-species level. These findings were consistent with those of Scott *et al* (in press) who used PyMS to examine phenotypic variation in populations of *Fucus spiralis*. PyMS analysis

was congruent with morphological data that two morph types existed; these were subsequently designated as *Fucus spiralis* (normal type) and *F. spiralis forma nanus* (dwarf form) (Scott *et al.*, in press).

PyMS was shown to be robust to the influence of environmental variability in fucoid algae to a limited degree. Separation of species was achieved; however, PyMS analysis did not always result in clear separation of *Fucus spiralis* and *Fucus vesiculosus* (Fig. 4.8). Introgression of these two species has been documented (e.g. Scott & Hardy, 1994); thus it may be concluded that PyMS data presented here reflects genetic, as well as phenotypic, variability. PyMS-derived similarity dendrograms were not entirely reproducible as the relative positions, within the dendrogram, of the species examined were not consistent for all shores. The use of PyMS as a tool for taxonomic investigations in marine macroalgae has thus far produced conflicting results. Hardy *et al* (1998) also demonstrated separation of species within the genus *Fucus* using PyMS, however, their results suggested that *F. serratus* and *F. vesiculosus* were more similar to each other than either of them was to *F. spiralis*. The results presented here suggest that a greater degree of similarity exists between *F. vesiculosus* and *F. spiralis* than between either of these species and *F. serratus*. It is suggested that the use of PyMS as a taxonomic technique for macroalgae should therefore be treated with caution. PyMS clearly provides a measure of chemical differences among samples, but to what extent these data contribute reliably to the classification of macroalgae is uncertain.

6.1.3 Environmental variability II – Laboratory investigations of diatom cultures

The adaptive response of *S. costatum* to variations in environmental condition is well established (e.g. Brzezinski, 1985; Yamomoto & Tsuchiya, 1995; Bienfang *et al.*, 1982). PyMS detected chemical differences among *Skeletonema costatum* cultured under variable salinity and silicate

concentrations (Figs. 5.2 – 5.3) and variable irradiance (Fig. 5.4). The use of axenic cultures *of S. costatum* throughout for experiments reported in chapter 5 eliminated the possibility that differences detected by PyMS were due to sample contamination. Therefore PyMS differences among cultures were attributed to environmentally induced variation as a result of culture condition. The precise nature of chemical differences among cultures detected by PyMS could not be determined, however the discrimination shown here further illustrates the ability of PyMS to detect variations at the sub-species level and highlights its potential as a sensitive technique. The effect of experimentally induced variation on PyMS data reported here shows some agreement with the findings of Freeman *et al.* (1994b) who showed significant within-strain spectral variation in bacterial cultures resulting from phenotypic differences among sub-cultures.

6.2 Further development and future applications

In many studies of natural ecosystems there is a need for a rapid, accurate technique, of high discriminatory power, for the characterisation of large numbers of isolates. The results presented here suggest that phenotypic and chemical variability among samples can be detected by PyMS analysis. That PyMS can be used for the analysis of multi-cellular organisms has also been shown by this study. It is also sufficiently sensitive to allow resolution of chemical differences among isolates at a small sample size, provided that sample concentration is suitable.

A limiting factor of PyMS as a technique for use in algal systematics is not the discrimination itself, but its ability to overcome the inherent variability among samples from the environment. This variability may originate from sample contamination, stage in life history, or the developmental state of the organism. Any multi-cellular or unicellular organism is a unit with component parts. If such an organism is considered as a complex "cell", then it may be possible

to resolve some of these difficulties. One way of doing this is to analyse components of the cell as opposed to the whole-cell. For example, Kokinos *et al.* (1998) used PyMS as a method for the chemical characterisation of biomacromolecular material by analysis of solvent extract from marine dinoflagellate resting cyst walls. Since PyMS data reflect overall cellular composition and processes, it may also be of potential use as a tracing technique. The application of PyMS as a technique for the semi-quantitative analysis of the origins of marine particulate organic matter has recently been attempted with some success (Minor *et al*, 2000).

The main limitation in the application of PyMS as a comparative technique is the restriction of its use to that of resolution within-batch. However recent developments in data analysis methods have largely overcome this problem. One particularly important development in the analysis of PyMS data is the application of artificial neural networks (ANNs) to the deconvolution of spectral information. ANNs are a novel approach to analysis of non-linear relationships in multivariate data, such as those generated by PyMS, and have been reviewed by Goodacre (1994). Correction of pyrolysis mass spectral drift has also been achieved through ANN analysis by relating the spectral data from one run (input), to the spectral data from a second run (output), and using the resulting model to predict the target of unclassified spectra (Goodacre & Kell, 1996a). Interlaboratory reproducibility has also been achieved using ANNs to transform the spectral data from one instrument to match PyMS spectra of a second instrument using an appropriate algorithm (Goodacre *et al.*, 1997). This is a development which has important implications for the future use of this technique. For example, the combination of PyMS and ANNs has been used to detect the toxin production status of strains of *Escherichia coli* (Sisson *et al.*, 1995).

The use of ANN analysis techniques with PyMS data is likely to provide the greatest potential for the routine use of this technique in marine algal systematics. For example, PyMS combined with ANN analysis may be a suitable method for the rapid screening of toxin and non-toxin producing strains of algae, including diatoms and dinoflagellates. Analysis of PyMS data using ANNs is likely to be of value for the rapid screening of ecologically important multi-species complexes such as phytoplankton. The successful use of ANNs for analysis of marine phytoplankton from flow cytometry data has been reported (Wilkins *et al.*, 1996). Flow cytometry is an established automated technique for the analysis of phytoplankton establishing size frequency data for the multi-sized populations of particles. Wilkins *et al* (1996) suggested that the combination of the flow cytometric approach with an equally rapid classification method would provide the greatest potential for its routine use. The rapid, automated and highly discriminatory nature of the PyMS technique and the applicability of ANNs to analysis of PyMS-derived data may qualify this method for combination with techniques such as flow cytometry.

6.3 Conclusions

PyMS has potential value as a rapid screening technique for marine algal systematics but is currently subject to technical constraints, namely limited day to day reproducibility and the influence of variation among samples. However this study has shown that PyMS is a highly discriminatory technique that can be used to rapidly detect small biological and chemical variation among samples. PyMS is not a substitute for biochemical or DNA-based molecular methods, nor the use of traditional taxonomic methods, but compliments these existing, highly specific analytical techniques. PyMS is useful as a tool for the integrated use of genotypic and phenotypic data, known as polyphasic taxonomy, for systematic studies. The future development

of robust data analysis methods for PyMS data, particularly artificial neural networks, will have important implications for the use of this technique.

Appendix

Appendix 1

'F/2 + Si' medium (Guillard's medium for diatoms)

	Amounts per 1000 cm^3
$NaNO_3$	0.075 g
$NaH_2PO_4.2H_2O$	0.00565 g
Trace elements stock solution (1)	1.0 cm^3
Vitamin mix stock solution (2)	1.0 cm^3

Make up to 1000 cm^3 with filtered natural seawater

Adjust to pH 3.0 – 4.30 with HCl

Add sodium metasilicate stock solution (3) while stirring 3.0 cm^3

Adjust to pH 8.0 with 1M NaOH or HCl

Stock Solutions:

(1) Trace elements	**Amounts per 1000cm^3**
Na_2EDTA	4.360 g
$FeCl_36H_2O$	3.150 g
$CuSO_4.5H_2O$	0.010 g
$ZnSO_4.7H_2O$	0.022 g
$CoCl_2.6H_2O$	0.010 g
$MnCl_2.4H_2O$	0.180 g
$Na_2Mo_4.2H_2O$	0.006 g

(2) Vitamin mix	**Amounts per 1000 cm^3**
Cyanocobalamin (Vitamin B_{12})	0.0005 g
Thiamine HCl (Vitamin B_1)	0.1 g
Biotin	0.0005 g

(3) Sodium metasilicate	**Amounts per 1000 cm^3**
$Na_2SiO_3.5H_2O$	100 g

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